ALLEN'S COMMERCIAL
ORGANIC ANALYSIS

A TREATISE ON

THE PROPERTIES, MODES OF ANALYSIS, AND PROXIMATE ANALYTICAL EXAMINATION OF THE VARIOUS ORGANIC CHEMICALS AND PRODUCTS EMPLOYED IN THE ARTS, MANUFACTURES, MEDICINE, ETC.

WITH CONCISE METHODS FOR

THE DETECTION AND ESTIMATION OF THEIR IMPURITIES, ADULTERATIONS, AND PRODUCTS OF DECOMPOSITION

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VOLUME X

Haemoglobin and its Derivatives, Albuminoids or Scleroproteins, Structural Proteins, Examination of Foodstuffs for Vitamins, The Hormones The Identification of Unknown Woods and Charcoals, The Pectic Substances

BY THE EDITOR AND THE FOLLOWING CONTRIBUTORS

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ALLEN'S
COMMERCIAL ORGANIC ANALYSIS

FIFTH EDITION, REWRITTEN, REVISED, RESET

COMPLETE IN TEN VOLUMES EACH VOLUME SOLD SEPARATELY

The organic chemicals and products employed in the arts, manufactures, commerce, medicine, science, etc. It treats upon the properties, modes of analysis, proximate analytical examination; methods for detection and estimation of impurities, adulterations, products of decomposition, etc.

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PREFACE

This volume completes the Fifth Edition of *Allen's "Commercial Organic Analysis,"* and it has, of necessity, become more miscellaneous in character than the preceding volumes. Three of the sections on Proteins, which had to be held over from Volume IX, now come first. As they were too short for a whole volume, it was thought that the work could be made most useful by adding sections on subjects which have come into prominence during the time since this edition was begun. These sections comprise contributions on Vitamins, on the Hormones, on the Identification of Unknown Woods and Charcoals, and on the Pectins. Lastly, a General Reference Index of the whole ten volumes has been added, and, although space did not permit of this including an Authors' Index, yet many names associated with special processes have been given.

Of those who contributed to the last edition, Messrs. Gardner and Buckmaster and Mr. Alexander have revised their respective sections. The section on *Fibroids*, formerly contributed by Mr. Dreaper, has been revised by Mr. R. H. Marriott, who has added a large amount of new material, including the examination of furs. This section now appears under the title of *Structural Proteins*. The names of the other contributors appear for the first time.

C. Ainsworth Mitchell.

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HAEMOGLOBIN AND ITS DERIVATIVES

BY JOHN ADDYMAN GARDNER, M.A. (OXON), F.I.C.,

AND

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Blood, as it circulates in the vessels of man and other vertebrates, is a viscid liquid of a homogeneous red colour to the naked eye, of a bright scarlet hue in arterial blood, dusky red or claret-coloured in venous blood. Blood prevented from coagulating separates into a dark sediment formed of red corpuscles, each of which consists chiefly of a red colouring matter, ***hæmoglobin;*** the pale straw-coloured supernatant liquid is *liquor sanguinis*, or ***blood-plasma.*** Shortly after leaving the body, usually within 2 to 6 minutes, unless precautions are taken to prevent this, blood commences to coagulate; a pellicle of fibrin forms on the surface, this extends along the side of the containing vessel, and finally, in about ten minutes, the liquid is converted into a solid jelly. The process of coagulation is due to the formation of *fibrin* from fibrinogen, a protein of the plasma. The fibrin as it forms entangles the blood corpuscles. The jelly-like mass now shrinks, and a straw-coloured fluid, ***blood serum,*** exudes from the clot. Finally, at the end of 24 hours, the shrunken clot, often a third of its original bulk, remains adherent to the wall of the vessel, sunk in a quantity of serum. In sterile vessels this shrinking often occurs to only a small extent (Lister), therefore the yield of anti-toxic sera from blood collected in sterile flasks may be small. If shed blood is stirred with a bundle of wires or feathers for some minutes, as fast as the fibrin forms it collects on the wires. In this way all the fibrin, as it is formed, may be removed from blood during the process of coagulation. A red liquid is left; this is defibrinated blood—a suspension of corpuscles in serum.

Circulating blood = plasma + corpuscles (red corpuscles and leucocytes).

Clotted blood = serum + clot (fibrin and corpuscles).

Stirred blood = defibrinated blood (serum and corpuscles) + fibrin.

Reaction of Blood.—According to the indicator employed, the reaction of blood is either acid or alkaline (B. Moore, *Proc. Roy. Soc.*, 1905, 76B, 138). To litmus, methyl-orange or dimethyl-aminoazo-benzene, blood-plasma is alkaline. To phenolphthalein it is acid. The grade of alkalinity, chiefly due to hydrogen disodium phosphate, determined by titration with tartaric acid (Engel, Loewy, Zuntz) or

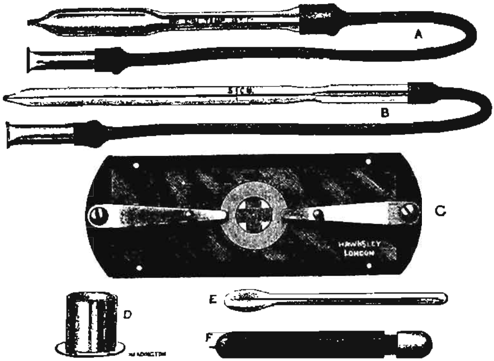


FIG. 1.

sulphuric acid (A. Wright, *Lancet*, 1897, Sept. 18) is equivalent to a solution of 0.4% sodium hydroxide (Loewy) after the corpuscles have been broken up.

The sp. gr. of defibrinated blood is best determined by some form of pycnometer, and in human adults averages 1.058, but can vary between 1.045 and 1.075. For drops of fresh human blood by Hammerschlag's method, in 165 adult males the average was 1.0544. In 149 of these the variation was 1.050–1.065; in 57% of the whole from 1.054–1.060; in 4 from 1.046–1.049; in 9 from 1.068–1.070, and in 3 cases only 1.040–1.042 (G. N. Stewart, *Manual of Physiology*, 5th ed., 1906, page 19).

Hammerschlag's Method.—A drop of blood ejected from a capillary tube under the surface of a mixture of chloroform (sp. gr. 1.498)

and benzene (sp. gr. 0.874) becomes a sphere. By cautious addition and mixing, either chloroform or benzene is added until the blood-drop is suspended in the mixture. The sp. gr. of this liquid, taken with the hydrometer or sp. gr. beads, gives the sp. gr. of the blood. Instead of benzene Inchley (*Proc. Physiol. Soc.*, Aug., 1904) suggests the use of Pratt's petrol A.

Enumeration of Red Corpuscles.—(1) *With Gowers' hæmacytometer* (*Lancet*, Dec. 1, 1877) (Fig. 1).[1]

A is a small pipette, which, when filled to the mark on its stem, holds exactly 995 mm.^{3}; B, a capillary tube marked to contain

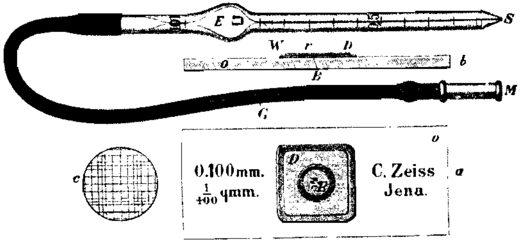


FIG. 2.—Mixing pipette and counter for red blood-corpuscles. (*After*) *Thoma.*

exactly 5 mm.^{3}; and C, a brass stage-plate, carrying a glass slip, on which is a cell 0.2 mm. deep. The bottom of this is divided into squares 0.1 mm. in diameter. Upon the top of the cell rests a cover-glass, which is kept in its place by the pressure of two springs proceeding from the ends of the stage-plate. D is a small glass jar, E, a glass stirrer, F, a sharp spear-pointed lancet.

In using the hæmacytometer, 995 mm.^{3} of an aqueous solution of sodium sulphate of 1,025 sp. gr. is sucked up by the pipette, A, and blown into the mixing jar, D. The finger, carefully cleaned with acetone, is then pricked with the lancet, the first drop of blood wiped away, and 5 mm.^{3} of the second drop of blood sucked into the capillary pipette, B, and blown into the diluting liquid. The two liquids are well mixed by rotating the glass stirrer, E, between the finger and

[1] Gowers' Hæmacytometer is made and sold by Messrs. Hawksley & Sons, 357 Oxford Street, London, W., to whom the authors are indebted for the illustration in the text.

thumb, and a small quantity of the diluted blood placed in the centre of the glass-cell, a cover-glass gently placed on the top, and secured with the brass springs, which should be *lifted* and not slid into position. The plate is then placed on the stage of a microscope. In a few minutes the corpuscles will sink to the bottom of the cell, and, on focussing, can be seen to settle on the squares. The corpuscles in ten of the squares toward the centre of the cell are then counted, and their number, multiplied by 10,000, gives the number in a cubic millimetre of the blood examined. In each square, for normal blood, there is an average of about 50 corpuscles.

(2) *With the Thoma-Zeiss Hæmacytometer.* (Fig. 2.)

The two parts of this apparatus are (*a*) a *counter.* Various forms may be used, such as the Thoma-Zeiss counter, Zappert counter, or Bürker counter; that of Thoma-Zeiss is a glass slide with a square affixed, so as to form a cell. Cemented to the floor of the cell and of less diameter is a circular disc B with ruled squares, a portion of which as seen under the microscope is shown at C; the area of each square is $1/400$ sq. mm. When accurately covered with a flat square cover-glass, this ruled disc lies exactly 0.1 mm. from the under surface of the cover, and the volume of the column of liquid standing upon a square is therefore $0.100 \times \frac{1}{400} = \frac{1}{4{,}000}$ mm.^{3} (*b*) *A Mixing Pipette.*—The capillary of this is expanded into a bulb E between the marks 1 and 101, containing a glass bead. Allowance is made for the size of bead, and the divisions on the capillary bear an exact ratio to the volume of the bulb. Thus at 05 or 1 the volume in the capillary is to the bulb when filled up to mark 101 as 1:200 or 1:100. A second pipette which allows of dilutions of 1:10 is used for enumeration of leucocytes, and the two are often supplied in one case.

To make a blood-count, suck up the blood, using the second drop which exudes from a puncture on the skin which has been cleaned with acetone (root of finger-nail or ear) to the mark 05 or 1; wipe the ends of pipette clean with filter paper, and then with the pipette immersed in the diluting fluid[1] suck this up slowly while the pipette is continuously rotated between the thumb and finger so as to mix well. Stop when the liquid exactly reaches the 101 mark. With-

[1] Diluting fluids for red corpuscles: Filtered Hayem's fluid: $HgCl_2$ 5 grm., Na_2SO_4 5 grm., NaCl 1 grm., H_2O 200 c.c. For white corpuscles: 3 % glacial acetic acid in water with 1 % of 1 % aqueous solution of Gentian-Violet or Methyl-Violet.

out removing the rubber tube, G, close with the fingers both ends of pipette, M and S, and shake vigorously for 1 minute. Next, by blowing, eject 3 or 4 drops, wipe end of pipette, and now blow out a minute drop on to the centre of the ruled disc of the counter. Cover carefully with the plane cover-glass. In doing this the liquid should not escape into the circular well round the disc, and Newton's rings should be seen as an interference phenomenon when the cover is accurately in contact with the square cell. Count the corpuscles in a number of squares, also including those which lie on the base line and left side of each square. Corpuscles which lie on the top and right-hand side do not belong to the square. In counting, count five squares left to right, next lower set right to left, then five below, left to right, and so on. As you proceed, note on paper the totals for each 5 or 10 squares. At least 40 squares should be counted. The number of corpuscles per cubic millimetre of blood is given by

$$\frac{4{,}000 \times \text{degree of dilution} \times \text{total corpuscles counted}}{\text{No. of squares counted}}$$

In sucking blood into the capillary, it is not necessary that the mark 05 or 1 should be exactly reached. Thus at 03, 05.4, 06, the ratio of blood volume to the bulb is 03/1,000, 05.4/1,000, 06/1,000, . . . and the degree of dilution is 1,000/03, 1,000/05.4, 1,000/06 . . .

Abbe has shown that when 16 squares are counted the error is 5%, when 100 about 2%, when 400 about 1%. However, a variation of 100,000 red corpuscles per cubic millimetre has no physiological interest.

Average number of red corpuscles per cubic millimetre of blood. *Man*, 5,000,000; *dog*, 6,500,000; *cat*, 9,000,000; *pig*, 6,000,000 to 8,000,000; *horse*, 7,700,000; *ox*, 6,000,000 to 8,000,000; *sheep*, 10,000,000; *goat*, 13,000,000 to 18,000,000.

Average Size of Red Corpuscles.—In the blood of all the mammals, except the *Camelidæ* which are oval, these are circular biconcave discs. The corpuscles of the blood of birds, reptiles, amphibia, and fish are oval (except in the lamprey), nucleated, and larger than mammals.

The average size of the blood-corpuscles from each species is approximately constant. Thus the corpuscles of human blood have an average diameter of about 1/3,200 in., but in fresh human blood,

corpuscles may always be found which vary very considerably from the *mean* value. The corpuscles of the human fœtus are usually larger, averaging 1/3,000 in., and occasionally reaching nearly twice the diameter of adult human blood. At birth a few nucleated red corpuscles may always be found.

The following table shows the diameter of the red corpuscles of various mammalia, expressed both in fractions of an inch and in decimals of a millimetre. The blood was in each case allowed to dry in a thin layer on the slide. (Wormley, *Michrochemistry of Poisons*, Philadelphia, 1888; Gulliver, *Proc. Zool. Soc., Lond.*, 1875, 474.)

Source of blood	T. G. Wormley		Gulliver	
	Inch	Millimetre	Inch	Millimetre
Man	1/3,250	.0078	1/3,200	.0080
Man, fœtus	1/3,000	.0085	....	....
Cat	1/4,372	.0058	1/4,404	.0058
Dog	1/3,561	.0071	1/3,532	.0072
Fox	....	....	1/4,177	.0061
Pig	1/4,268	.0059	1/4,230	.0060
Elephant	1/2,738	.0093	1/2,745	.0092
Horse	1/4,243	.0059	1/4,600	.0054
Ass	1/3,620	.0070	1/4,000	.0064
Ox	1/4,219	.0060	1/4,267	.0059
Sheep	1/4,912	.0052	1/5,300	.0048
Goat	1/6,189	.0041	1/6,366	.0039
Deer	....	....	1/7,060	.0036
Hare	....	....	1/3,560	.0071
Rabbit	1/3,653	.0069	1/3,607	.0070
Mouse	1/3,743	.0067	1/3,814	.0066
Rat	1/3,652	.0069	1/3,754	.0067

The corpuscles of the blood of some of the quadrumana are very similar in size to human corpuscles. Gulliver has recorded the following measurements, in fractions of an inch.

Lemur anguanensis,	1/3,976; 1/4,003; 1/4,440.
Ateles ater,	1/3,368; 1/3,342; 1/3,412.
Chimpanzee,	1/3,512; 1/3,602.

The following are measurements by Gulliver of the corpuscles of the blood of certain oviparous vertebrate animals. The figures are in fractions of an inch.

Source of blood	Longer diameter	Shorter diameter
1. Fowl	1/2,102	1/3,466
2. Turkey	1/2,045	1/3,508
3. Pigeon	1/1,973	1/3,643
4. Duck	1/1,937	1/3,424
5. Goose	1/1,836	1/3,839
6. Turtle (green)	1/1,231	1/1,882
7. Frog	1/1,108	1/1,821
8. Toad	1/1,043	1/2,000
9. Trout	1/1,524	1/2,460
10. Pike	1/2,000	1/3,555
11. Eel	1/1,745	1/2,842

Composition of the Red Corpuscles.—For the dog Alderhalden (*Zeitsch. physiol. Chem.*, 1898, **25**, 88) gives for 1,000 parts by weight:

Water	644.25
Solids	355.75
Hæmoglobin	327.52
Proteins	9.918
Cholesterol	2.155
Lecithin	2.568
Fatty acids	0.088
Phosphoric acid as nuclein	0.110
Na_2O	2.821
K_2O	0.289
Fe_2O_3	1.573
MgO	0.071
Cl	1.552
P_2O_5	1.635
P_2O_5 (inorganic)	1.298

According to Brandenburg, 1,000 parts by weight of red corpuscles contain the following amounts of K_2O: *Cat*, 0.058, *dog*, 0.257; *man*, 4.294; *horse*, 4.957; *rabbit*, 5.229.

From the above table it will be seen that hæmoglobin, as it is known to the chemist, when separated from the corpuscles cannot be held in solution in the red corpuscles, for 32% of hæmoglobin cannot be in solution with 62% of water, since at 37° C. only 18% hæmoglobin can be held by water.

Laking of Blood.—When blood is treated with distilled water, both hæmoglobin and the electrolytes may leave the corpuscles. The liquid becomes transparent and is said to be *laked*, or the corpuscles to be hæmolysed. Within the body hæmolysis may occur. The hæmoglobin then passes into the plasma and may be excreted in the urine. The term hæmaturia indicates that blood corpuscles

exist in urine; hæmoglobinuria that hæmoglobin is present in solution. Variable amounts of methæmoglobin are nearly always present in cases of hæmoglobinuria. Normal urine often contains traces of aporphyrin. Intermittent hæmoglobinuria is not uncommon in individuals suffering from malaria, and blood may originate from hæmorrhage of the kidney, bladder or urethra. The mode of action of hæmolytic agents varies, but the phenomenon is produced by:

1. Distilled water.

2. Grinding up corpuscles with sand (Rywosch).

3. Substances such as urea, ammonium chloride, glycerol, alcohol, ether, bile salts.

4. Glucosides, such as saponin, cyclamin, githagin, and solanin (Kobert).

5. The toxins of bacteria such as tetanolysin of *Bacillus tetani*, megatheriolysin of *Bacillus megatherium*, or the toxic lysins of *Staphylococcus* or *Bacillus pyocyaneus*.

6. Not all, but many snake-venoms cause hæmolysis both inside and outside the body, *e. g.*, those of the cobra, rattle-snake, water-moccasin, copperhead, Daboia and Enhydrina.

7. The blood serum of one animal is often hæmolytic for the blood of a different species, *e. g.*, that of tbe dog for the rabbit. The serum of the eel (Mosso 1888) and of the frog (Krompecher, Buckmaster) is hæmolytic for most kinds of mammalian blood.

8. Abrin, ricin and robin are phyto-albumins which produce hæmolysis.

9. Hæmolytic sera may be experimentally obtained in a variety of ways (Belfanti and Carbone 1888, Bordet 1888, Ehrlich and Morgenroth (1899). Thus, by repeated injections of guinea-pig's blood into the rabbit, the serum of the rabbit becomes hæmolytic for the red corpuscles of the guinea-pig. Similarly for goat's blood into rabbits, the rabbit serum is found hæmolytic for goat's corpuscles. This type of experiment can be varied for many animals, the blood being injected into the rabbit, and specific hæmolytic sera be obtained by this injection of foreign blood corpuscles (v. precipitation test for blood, page 110). The substances in sera which cause hæmolysis are known as hæmolysins.

The blood of fishes appears to differ essentially from that of mammals, as it cannot be laked by any of the ordinary methods

without clotting. This probably points to the existence within the corpuscles, and perhaps in combination with the hæmoglobin, of proteins which have no equivalent in warm-blooded organisms.

A number of drugs introduced into the body of man or animals cause not only hæmolysis but a conversion of oxyhæmoglobin into methæmoglobin, and in this latter form the pigment appears in the urine, so that this may appear almost black. Such drugs are: (1) Chlorates of potassium and sodium; (2) nitrites and nitrobenzene; (3) phenylhydroxylamine; (4) aniline derivatives in phenacetin, acetanilide; (5) quinine, according to R. Koch, Plehn and others, induces those attacks of black-water fever which are seen in tropical countries where a pernicious type of malaria is endemic. 5 grains of quinine will often be sufficient to cause the appearance of methæmoglobin in the urine of patients with malaria.

Blood, even in a thin layer, is opaque, dark when examined by transmitted light and clear by reflected light. From this fact alone it is obvious that blood in this optical property resembles those colours which are not true solutions but fine suspensions of colouring matter in a colourless medium, and hence it could be inferred that the blood-colouring matter is not in true solution. The colouring matter of the blood of vertebrate animals is a complex iron-containing protein, which can be obtained in a crystalline form. This is the chief constituent of the red corpuscles, and, owing to its presence, they act as the carriers of oxygen in the organism. It possesses the remarkable quality of linking to itself a molecule of oxygen to form an easily dissociable compound, known as *oxyhæmoglobin* ($Hb + O_2$); but oxyhæmoglobin and hæmoglobin (reduced hæmoglobin) exist side by side in various proportions in circulating blood, the former being more abundant in arterial, the latter in venous blood. Hæmoglobin is a protein, easily decomposed by moderate heat, weak acids or alkalies, into *globin* (a histone) and a relatively simple group containing the iron (hæm) coloid in combination with denatured globin forms a crystallizable substance spoken of as *hæmochromogen*, which easily unites with oxygen and is then known as *hæmatin*. The hydrochloride of this is crystalline *hæmin*. Removal of the iron from either of these three substances by strong acids yields coloured iron-free derivatives; of these *hæmatoporphyrin* and *hæmatoidin* are examples.

TABLE 1.—ELEMENTARY ANALYSIS OF OXYHÆMOGLOBIN

Source	C.	H.	O.	N.	S.	Fe.	P.	Observer	Reference
Dog	53.64	7.11	21.02	16.19	0.66	0.43	0.91 (P_2O_5)	C. Schmidt	Hoppe-Seyler's *Med. Chem. Unter.* 1867, **2,** 169.
Dog	53.85	7.32	21.84	16.17	0.36	0.43	...	F. Hoppe-Seyler	*Ibid.*, 1867, **2,** 189.
Goose	54.26	7.10	20.69	16.21	0.54	0.43	0.77 (P_2O_5)	F. Hoppe-Seyler	*Ibid.*, 1867, **2,** 194.
Guinea-pig	54.12	7.36	20.68	16.78	0.58	0.48	...	F. Hoppe-Seyler	*Ibid.*, 1868, **3,** 370.
Squirrel	54.09	7.39	21.44	16.09	0.40	0.59	...	F. Hoppe-Seyler	*Ibid.*, 1868, **3,** 370.
Horse	54.87	6.97	19.73	17.31	0.65	0.47	...	Kossel	*Z. physiol. Chem.*, 1878, **2,** 150.
Dog	54.00	7.25	21.45	16.25	0.63	0.42	...	J. Otto	*Ibid.*, 1882, **7,** 61.
Pig	54.17	7.38	21.36	16.23	0.66	0.43	...	J. Otto	*Ibid.*, 1882, **7,** 61.
Horse	54.76	7.03	19.81	17.28	0.67	0.45	...	J. Otto	*Pflüger's Arch.*, 1883, **31,** 243.
Horse	54.40	7.20	19.67	17.61	0.65	0.47	...	M. Bücheler	*Dissert.*, Tübingen, 1883.
Horse	51.15	6.76	23.43	17.94	0.39	0.34	...	O. Zinoffsky	*Z. physiol. Chem.*, 1885, **10,** 32.
Ox	54.66	7.25	19.54	17.70	0.45	0.40	...	G. Hüfner	*Ludwig's Festgabe*, 1890, **80.**
Pig	54.71	7.38	19.60	17.43	0.48	0.40	...	G. Hüfner	*Ludwig's Festgabe*, 1890, **80.**
Dog	53.91	6.62	22.62	15.98	0.54	0.33	...	A. Jaquet	*Z. physiol. Chem.*, 1888, **12,** 288.
Dog	54.37	7.22	21.93	16.38	0.57	0.34	...	A. Jaquet	*Z. physiol. Chem.*, 1889, **14,** 291.
Fowl	52.47	7.19	22.50	16.45	0.86	0.34	0.20	A. Jaquet	*Z. physiol. Chem.*, 1889, **14,** 295.
Horse	54.56	7.15	.....	17.33	0.43	....	...	F. N. Schulz	*Z. physiol. Chem.*, 1898, **24,** 469.
Horse	54.75	6.98	20.12	17.35	0.42	0.38	...	E. Alderhalden	*Z. physiol. Chem.*, 1903, **37,** 494.
Turtle	54.77	6.99	20.38	17.07	0.38	0.46	...	Bardochezi	*Z. physiol. Chem.*, 1906, **49,** 466.
Cat	54.60	7.25	20.66	16.52	0.62	0.35	...	Abderhalden	*Lehrb. d. physiol. Chem.*, 5 ed. p. 688 (Berlin, 1923).

Occurrence.—Hæmoglobin and allied colouring matters are found in animals[1] either (1) in special morphological elements of the blood or perivisceral fluids, (2) in a state of solution in the liquids of the body, (3) in muscular tissue, (4) in nervous tissue, (5) in the urine and fæces (pathological).

The hæmochromogen from hæmoglobin appears to be identical in all vertebrates, though the blood-colouring matter itself exhibits differences in physical characters and chemical composition. The published elementary analyses of crystallised purified oxyhæmoglobin, dried between 100–110°, given by various observers, do not completely agree, as will be seen in Table 1.

The globin of hæmoglobin has generally been regarded as a histone, but the presence of only 20% of diamino-acids is against this view; further, the principal diamino-acid is histidine (β-iminazole-α-amino-

TABLE 2.—PRODUCTS OF THE HYDROLYSIS OF THE HISTONE MOIETY OF HÆMOGLOBIN FROM HORSES' AND DOGS' BLOOD, ACCORDING TO ABDERHALDEN[1]

Products of hydrolysis	Globin of horses' blood	Globin of dogs' blood
Glycine	....	....
Alanine	4.19	3.0
Valine	....	1.0
Leucine	20.04	17.5
Phenylalanine	4.24	5.0
Tyrosine	1.33	....
Serine	0.56	....
Cystine	0.31	....
Proline	2.34	4.5
Oxyproline	1.04	....
Aspartic acid	4.03	2.5
Glutamic acid	1.73	1.2
Tryptophane	+	....
Arginine	5.42	....
Lysine	4.28	....
Histidine	10.96	....
Total	69.87	34.7

[1] *Z. physiol. Chem.*, 1903, **37**, 454; 1907, **51**, 397.

propionic acid), whereas in other histones it is arginine (α-amino-δ-guanidine-valeric acid).

[1] Preyer, "*Die Blutkrystalle,*" p. 6. E. Ray Lankester, *Proc. Royal Soc. Lond.*, 1872, **21**, 70–81. R. Hertwig, *Lehrb. v. Zool.*, 6 Auf., Jena 1903. O. v. Fürth, *Vergleich. Chem. Phys. v. nied. Tiere*, Jena 1903. P. Nolf, Art. *Hæmoglobine*, Richet's *Dict. de Physiol.*, 1908, page 321. Gamgee, *Textbook of Physiology*, ed. by Schäfer, 1898, **1**, page 156.

On the assumption that the molecule of hæmoglobin contains only 1 atom of iron and that the percentage of iron is 0.336, the molecular weight of oxyhæmoglobin is 16,662, and calculating from the fact that 1 grm. of hæmoglobin from ox blood unites with 1.338 c.c. of CO at 0° and 760, and assuming that Hb and CO unite in molecular proportion, the molecular weight would be 16,736.

The molecular weight has been determined from the osmotic pressure. Assuming that 1% solution of oxyhæmoglobin is undissociated and, as shown by the ultra-microscope, a true solution, an osmotic pressure of 10.77 mm. of mercury corresponds to a molecular weight of 16,669. After three recrystallisations without alcohol, Hüfner (*Arch. Anat. u. Physiol.*, 1907, **209**) for oxyhæmoglobin of the horse found a molecular weight between 14,630 and 15,840, the mean of 4 experiments gave 15,115; for oxyhæmoglobin from the ox 15,500 and 18,370, and the mean of 11 experiments 16,321. For 1% oxyhæmoglobin solution (Hüfner) the osmotic pressure in millimetres of mercury is,

Horse	Ox	Ox
11.90	10.18	11.03
11.14	10.61	10.45
11.64	10.59	9.31
12.10	10.67	10.55
......	10.78	10.29
......	......	11.00

The values found by Waymouth Reid (*J. Physiol.*, 1905, **33**, 12) for oxyhæmoglobin of the dog are only 3.5 to 3.8 mm. of mercury after one crystallisation, 4.1 to 4.3 mm. after a second crystallisation.

More recently G. S. Adair, (*Proc. Roy. Soc.*, 1925, A., **108**, 627) using the conductivity of the solutions as a test of their freedom from impurities, found that the solution of lowest conductivity gave an osmotic pressure of 3.2 mm. per 1% of protein at the iso-electric point. If the low values were due to aggregation, they should decrease still further at higher concentrations of protein, but the figures indicated a slight increase. Adair concluded that the molecular weight of hæmoglobin is about 4 times the equivalent. This conclusion is also supported by the work of Svedberg and Fahraens.

Preparation of Oxyhæmoglobin: Method of Preparation of Crystals in Small Quantities for Microscopic Examination

1. *Funke's method*, the simplest and oldest, for the blood of horse or rat, consists in mixing a drop of blood with a drop of distilled water on a slide by means of a needle. When the edges commence to dry, apply a cover-glass. Crystals form at once.

2. *Rollett's Method.*—Defibrinated blood is repeatedly frozen and thawed in a platinum crucible placed in a mixture of ice and salt or, better, snow and calcium chloride. After the last gradual thawing the laked blood is poured out into a glass vessel, forming a layer 15 mm. deep, and set aside in a cool place. In a short time crystals form, most easily from guinea-pigs' blood, and for other animals in the following order:—squirrel, cat, dog, man, rabbit, pig. Frog's blood yields no crystals by this method.

3. *Gscheidlen's Method.*—Defibrinated blood, which has been exposed to air for 24 hours, is sealed in narrow glass tubes (vaccine tubes) and then placed in the incubator at 37° for some days. On opening the tubes, the contents are spread on a slide and allowed slowly to evaporate. From the dog prismatic crystals, 3.5 cm. in length, may be obtained.

4. A drop of blood is allowed to dry somewhat at the edge, surrounded with a ring of Canada balsam dissolved in xylene, benzene, or chloroform, and then covered. Within half an hour crystals form.

5. Leeches which have sucked blood, some 3 weeks later are found frequently to have masses of hæmoglobin crystals in the crop.

Reichert states that when the blood of the dog, horse, rat, guinea-pig, or a cold-blooded animal like necturus, is laked with water, ether, or, best of all, ethyl acetate, and before or after laking ammonium oxalate to the amount of 1 to 5% added, hæmoglobin crystallises quickly and easily.[1]

S. M. Copeman (*J. Physiol.*, 1890, **11**, 401) found that if the blood from the finger of patients suffering from pernicious anæmia was allowed to fall on a glass slide and then, when the edge of the drop had dried somewhat, a coverglass was gently placed on it, crystals of hæmoglobin gradually formed in the film in 10 to 24 hours without any further preparation. After treatment with arsenic, the blood of such patients yielded no crystals. Bond found in cases of septic

[1] *Amer. J. Physiol.*, 1903, 9, 97. See also *The Crystallography of Hæmoglobin* by E. Tyson Reichert and A. P. Brown. *Publication* 116, *Carnegie Inst.*, Washington, 1900 (contains 100 plates illustrating crystals from different kinds of animals).

anæmia and pernicious anæmia, that hæmoglobin crystals formed spontaneously when blood was slowly dried. He also obtained crystals from normal human blood by mixing this with putrid human serum.

The most reliable method is that given by Copeman for the blood of the following animals: horse, bullock, sheep, pig, dog, cat, squirrel, rabbit, guinea-pig, rat, mouse and fowl. To defibrinated blood add 1/16 part of its volume of ether, and shake for some minutes until it becomes perfectly transparent or laky. Then set aside in a cool place for a period varying from a few hours to a few days (Gamgee, *Physiol. Chem.*, 1, p. 87). The shaking is done in a stoppered bottle, and the air allowed to escape gradually as the ether volatilises. By this means the air is replaced by ether vapour, while at the same time the small portion of blood which is forced out around the stopper of the bottle dries and so prevents ingress of air. The bottle is best kept at even temperature, not in a cool place. How long the blood should be left (in the case of most animals 2 days at least) is known by removing a drop, and then when the edges are slightly dry, gently lowering a cover-glass on to the surface. Crystals commence to form an hour or so later. Human blood mixed with putrid sheep's serum invariably yielded hæmoglobin crystals. These are in the state of reduced hæmoglobin.

Method of Preparation of Hæmoglobin Crystals in Quantity

Perfectly fresh defibrinated horse or ox blood is strained through muslin and centrifuged (2,000–2,400 revolutions per minute), the serum is poured off, and the deposit of blood corpuscles mixed with a volume of 0.85% sodium chloride equal to the volume of serum, and then again centrifuged. The sediment is then dissolved in the least possible quantity of distilled water at 37°. The solution is cooled to 0°, and mixed with half its volume of pure cold ether, transferred to a stoppered separating funnel and thoroughly shaken several times during the day. It is then allowed to stand for 24 hours or longer, when the content separates into three layers—the lower one a clear aqueous solution of hæmoglobin, a middle gelatinous layer with corpuscle residues, and an upper one of ether. The aqueous solution is run off into a beaker, and what is allowed to flow out must be free from the slightest turbidity. The solution is filtered into a large flask and freed from ether by drawing a current of air

through the flask. The air current must be purified by passing through concentrated solutions of potassium permanganate and concentrated sulphuric acid. The solution at 0° is now cautiously mixed, gently shaking the flask, with pure alcohol in proportions depending on the solubility of the hæmoglobin. For the horse the alcohol added equals one-fourth of the volume of the solution, for the ox one-third. All these operations must be conducted in a cold room, and the solution kept at 0° as nearly as possible. The whole is placed in a good freezing mixture (−20°) which is constantly renewed morning and evening. The hæmoglobin gradually crystallises out; for the horse in about 12 hours, for the ox in about 24 hours or longer. The crystals are separated from the mother-liquor by a centrifuge kept at 0°. They are then recrystallised without alcohol. The crystals are first washed with pure ice-cold boiled-out distilled water and then dissolved in the least possible quantity of pure boiled-out distilled water at 30°. They are then cooled to −3° and seeded with a small portion of washed crystals of the original crystallisation, and left to crystallise. The crystals are again separated by the centrifuge and the operation repeated. Gamgee (*Proc. Roy. Soc.*, 1902, **70**, 81) recommends thorough washing by decantation with 20% alcohol after each successive crystallisation and washing the ultimate product many successive times with large quantities of the purest distilled water at 0°. If the crystals are desired dry, the moist mass is spread out on a Schleicher and Schull thick filter-paper plate and dried *in vacuo* for 6 hours over sulphuric acid. The mass is powdered in an agate mortar and finally dried in a current of pure hydrogen in a flask kept at 105° in an air-bath or in the vapour of boiling toluene.

In the case of human blood it is not possible to crystallise the hæmoglobin by alcohol in the above manner, but crystallisation may be brought about by salting out with ammonium sulphate. The hæmoglobin obtained in this way is washed on the filter with ice-cold water and again dissolved (*Z. physiol. Chem.*, 1909, **62**, 193).

Dudley and Evans (*Biochem. J.*, 1921, **15**, 487) pointed out that excellent crystals are readily obtained by methods ordinarily in use for such blood as horse, but that it is rarely possible to bring the entire crystalline mass into solution in water. There is often an insoluble residue, indistinguishable from the soluble portion as far as crystalline character was concerned. They regard this as

due to denaturising effect of alcohol on protein. They give the following method in which they avoid denaturising agents: "Defibrinated horse blood is centrifuged and the corpuscles washed by the aid of the centrifuge with isotonic saline solution until the supernatant fluid gives no turbidity when heated to boiling. The thick corpuscular paste is then transferred to collodion tubes and dialysed under pressure of a column of mercury, first against running tap water for about 3 days, and finally against frequent changes of distilled water for about 2 days. The corpuscles are completely laked and the hæmoglobin partially reduced during this operation, a deep purple solution being obtained. This is centrifuged and can then be poured off from the deposit of stromata, etc. Oxygen is next bubbled through the clear solution until crystallisation of the oxyhæmoglobin occurs. As a rule, this is somewhat delayed, crystallisation suddenly taking place after about 20 minutes' oxygenation. The pasty mass is then centrifuged, the crystalline oxyhæmoglobin settling to the bottom of the tube as a thick scarlet paste. Under the microscope the crystals appear as very fine hair-like needles. The supernatant liquid is poured off. The crystalline oxyhæmoglobin so produced is *completely* soluble in water.

In order to recrystallise the material, it is suspended in two or three times its volume of water. The aqueous suspension is then warmed in the water bath to 37° C., and the containing flask is attached to a vacuum pump. Oxygen is pumped off and oxyghæmoglobin reduced to hæmoglobin, which, being much more soluble in water than the oxyhæmoglobin, gives a clear deep purple solution. This solution is cooled and re-oxygenated, when oxyhæmoglobin again crystallises out. The operation of re-crystallisation can be repeated as often as may be desired."

This, we think, is the best method of obtaining pure crystals from horse blood, but whether equally good crystals can be obtained from the blood of other animals the authors do not state.

The crystals of hæmoglobin of man and different animals belong, with one exception—the squirrel—to the rhombic system. In this animal they are hexagonal tables, but after many recrystallisations they change into rhombic needles and tetrahedra. Hæmoglobin crystals exhibit the phenomena of pleomorphism.

The water of crystallisation is usually determined in the following way. Pure hæmoglobin is dried *in vacuo* at 0°, and the water of

crystallisation driven off by heating to 115°. The accurate determination is difficult, but there can be no doubt that the crystals of hæmoglobin from different animals possess different amounts of water.

Oxyhæmoglobin	Water of crystallisation, %	Authorities
Dog	3·4	Hoppe-Seyler.
Horse	3·94	Hufner.
Pig	5·9	Otto.
Guinea-pig	6	Hoppe-Seyler.
Squirrel	9	Hoppe-Seyler.

Solubility.—The determination of the solubility of hæmoglobin is difficult, owing to the readiness with which the crystals undergo change, and also to the difficulty of avoiding traces of the reagents employed in the preparation of crystals. In the present state of knowledge, hæmoglobin from different animals differs markedly in solubility. In order to demonstrate the species specificity of haemoglobin, Landsteiner and Heidelberger made use of the well-known fact that the solubilities of substances which do not react on one another are additive. As a consequence, it would be expected that, if two samples of oxyhaemoglobin were different, each would dissolve in a saturated solution of the other as in pure water itself. On the other hand, if two preparations were identical, a saturated solution of one would obviously be saturated to both.

The solubility determinations were carried out at room temperature. 1 c.c. samples of the solution were used, and the content in hæmoglobin was determined by drying to constant weight *in vacuo* at 40°–50°. The freedom of their preparations from salts was controlled by conductivity measurements, and in some cases electrometric *p*H determinations were made on the solutions in order to make certain that the observed solubility effects were not due to differences in the *p*H of the solution. They found that the additive rule was applicable to the differentiation of the oxyhaemoglobins of not too closely related species. The oxyhaemoglobins of horse, rat, dog and guinea-pig show distinct differences when tested by this method. The oxyhaemoglobins of donkey and horse showed a similarity best explained by the assumption of isomorphism. The

hæmoglobin of man is most soluble in water, then follow in order that of the pig, ox, birds, horse, dog, squirrel, guinea-pig and rat. The crystals are soluble in highly diluted solutions of ammonia, alkali hydroxides and their carbonates. Such solutions resist decomposition much longer than pure aqueous solutions. Stronger solutions decompose hæmoglobin. The crystals are soluble in highly diluted alcohol, but by contact with this, they become less soluble in water. The crystals are insoluble in absolute alcohol.

Diffusibility.—Oxyhæmoglobin is a typical colloid in the sense of its absolute indiffusibility through animal membrane and parchment paper, but differs from most colloids in the facility with which it crystallises. When pure solutions of oxyhæmoglobin are subjected to electrolysis there occurs a separation of oxyhæmoglobin in a colloidal but perfectly soluble form. Gamgee, employing an electrolytic cell in which the anode is separated from the cathode by an animal membrane (sheep's intestine or pig's bladder), noticed that the first action of the current is to cause a separation of colloidal hæmoglobin in the anode cell. This colloidal hæmoglobin falls as a beautiful red cloud, leaving a perfectly colourless supernatant liquid. On stirring, it instantly dissolves. The further action of the current is to cause a rapid and entire transfer of the colloidal hæmoglobin from the anode to the cathode cell. With an electrolytic cell, of which each compartment had a width of 5 mm. and contained 2.5 c.c. of a 1% solution of oxyhæmoglobin, complete precipitation and transfer occurs within 60 minutes. On reversal of the current the hæmoglobin returns again in the direction of the positive current into the original cell from which it started. Gamgee considers that it is highly probable that within the corpuscle hæmoglobin is present in a colloidal form (*Proc. Roy. Soc.*, 1902, **70**, 83).

The conductivity of oxyhæmoglobin solutions is low. It increases rapidly with increase of temperature and undergoes remarkable and permanent changes when the solution is kept, even for short periods, at any temperature above 0°. It is therefore impossible to obtain very reliable data as to the absolute specific resistance of oxyhæmoglobin solutions. Gamgee (*Proc. Roy. Soc.*, 1902, **70**, 82), as the result of laborious investigations, gives the following numbers, expressed in reciprocal ohms, for the specific conductivity of solutions of oxyhæmoglobin.

Temp.	Solution of O_2Hb contains 3.07 % or 1 grm. mol. in 542,900 grm. Conductivity	Contains 2.235% O_2Hb or 1 grm. mol. in 745,800 grm. Conductivity
0°	$10^{-6} \times 2.626$	$10^{-6} \times 2.23$
18°	$10^{-6} \times 4.432$	$10^{-6} \times 3.25$
25°	$10^{-6} \times 5.19$	$10^{-6} \times 4.27$
39°	.. . ..	$10^{-6} \times 7.47$

Optical Properties of Oxyhæmoglobin.—As first described by Rollett and v. Lang, the crystals of oxyhæmoglobin exhibit a marked pleochroism. This is best seen with hæmoglobin and carboxy-hæmoglobin, less obviously with oxyhæmoglobin and methæmoglobin. This phenomenon is apparent when the crystals are rotated on a slide *on the stage of the polarising microscope, only the lower nicol being* used, and without an analyser in the eye-piece. Crystals of oxyhæmoglobin of the dog present a more or less permanent tint, according to their position with reference to the plane of polarisation. Some are scarlet-red, others orange or yellow. On rotation of the slide, each crystal passes insensibly from one to other of these colours. Hæmochromogen crystals also show pleochroism.

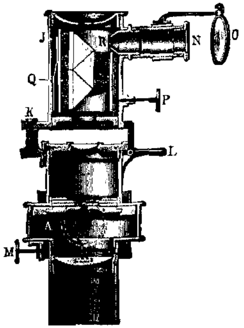
Examined with crossed nicols the crystals show double refraction. Those of the squirrel are an exception. Within the corpuscle hæmoglobin exhibits no double refraction. Pleochroism characterises the crystals more definitely than double refraction.

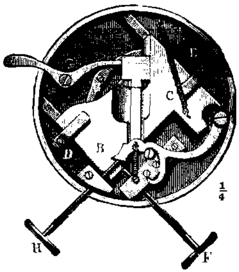
Specific Rotation.—Gamgee and Croft Hill determined this for oxyhæmoglobin and carboxyhæmoglobin. They filtered the light of an arc-lamp through a Landolt's filter, one compartment of which contained a solution of 0.05 grm. of hexamethylpararosaniline, dissolved in 1 litre of dilute alcohol, the second compartment being filled with 10% aqueous solution of potassium chromate. Only a narrow band of the spectrum passed through $\lambda 718\mu\mu$ — $\lambda 639\mu\mu$. *For oxyhæmoglobin the mean value was* $[\alpha]_c = +10°$. *For* Hb-CO$[\alpha]_c = +10.8°$. Globin, like other proteins, is lævo-rotatory $[\alpha]_c = -54.2°$.

Spectroscopic Properties of Oxyhæmoglobin.—The spectroscope commonly employed in physical and chemical laboratories may be used for studying the visible spectra of hæmoglobin and its derivatives. The absorption spectra of these are best investigated with an instrument provided with a single good flint glass prism rather than

with a train of prisms since, with the greater dispersion of the latter, the absorption bands are less clearly defined.

Dual vision spectroscopes of the Browning or Hilger patterns or direct vision spectroscopes, adapted to the eye-piece of the compound spectroscope, may be employed. This class of instrument may be utilised for the investigation of minute quantities of colouring matters. In Abbe's micro-spectroscope (Figs. 31 and 32) made by Zeiss the direct-vision prism can be moved away from or brought





The Zeiss micro-spectroscope.
FIG. 3.—Section of instrument. FIG. 4.—Mechanism of slit.

over the eye-piece at will, and is secured in position by means of a catch (L). By means of a screw and spring the micrometre-scale can be readily adjusted to the standard position with regard to the spectrum, namely, that in which the Fraunhofer-line D corresponds with the division 58.9. This arrangement allows the wave-lengths of all other lines and bands to be read off at once without the necessity of referring to a table. The length and width of the slit can be adjusted by the screws F, H.

For fine work, the spectroscope employed should be one furnished with means for determining accurately the position of any line or the boundaries of any absorption band observed in the spectrum, it being usual to express the position in terms of the wave-length of the light corresponding to it. For this purpose the spectrum of sunlight is

observed, and the position of the chief Fraunhofer lines in reference to the divisions of the graduated circle of the instrument is determined. From such observations a curve may be plotted, enabling the observer to convert the readings of the arbitrary divisions of his scale into wave-lengths. For accurate work, the eye-piece of the instrument should be provided with cross-threads, and for use in the investigations of absorption spectra, provided with a variable slit, such as is used in the spectrophotometer for isolating any desired region of the spectrum.

As a source of light, sunlight reflected from a white surface may be employed, or an incandescent burner. The latter is the best of all lamps for the examination of absorption spectra.

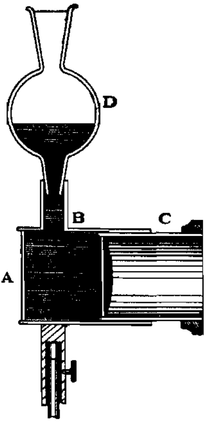


FIG. 5.

In examining the absorption spectra of liquids, these are placed in small cells with parallel glass or quartz sides which are a known width apart. Such vessels are known as hæmatinometers. The simplest is that devised by Hoppe-Seyler, and consists of a rectangular glass cell in which the internal surfaces of the larger glass plates are 1 cm. apart. In the hæmatoscope of Hermann (Fig. 5) the distance of the plates can be varied. The instrument consists of a glass plate A forming the anterior wall of tube B supported on a stand. The metallic tube C, sliding in front of tube B, is closed anteriorly by a glass plate parallel to A. A funnel D communicates with a space between the plates; by sliding the piston C in and out of B the distance between the two plates can be varied and the depth of the stratum of liquid read off on a millimetre scale engraved on C. With the hæmatoscope we can obtain, within limits, the same results with a solution of constant concentration as with a large number of solutions in which the concentration varies in known proportion, since the absorption of light passing through a coloured liquid depends upon the number of absorbing molecules in its path.

Specially characteristic of an absorption band is its point of *maximum absorption, which does not change with different concentrations* of the colouring matter, whereas the width of the bands changes very considerably. The point of maximal absorption of any band or bands in the spectra to be described later will be always indicated by the wave-length value of this region of maximal absorption.

In the case of hæmoglobin and its derivatives, some bands are visible with the naked eye and some can only be detected by using fluorescent eye-pieces or screens, or by special photographic methods: since these lie in or near the ultra-violet region of the spectrum. (For details of these methods see J. L. Soret, *Arch. phys. et nat. Geneva*, 1878, p. 322–357. A. Gamgee, *Proc. Roy. Soc.*, 1896, **59**, 256; Lewin, Miethe and Stenger, *Pflüger's Archiv.*, 1907, **118**, 80; *Beiträge zur Physiologie der Blutspectra;* Rost, Franz and Heise, *Arbeiten aus d. Kaiserlich. Gesundheitsamt*, 1909, **22**, Heft 12. Schumm, *Klinische Spectroskopie*, Jena, 1909; also article in Abderhalden's *Handbuch des Biochem. Arbeitsmethoden*, Band 6, p. 389. Mees, An Atlas of Absorption Spectra London, 1909. W. J. Dilling, *Die photographie der Blutspektra, Zeitsch. f. biolog. Tech. u. Math.*, 1912, Bd. II, No. 8, 353.) According to Gamgee, the bands in the ultra-violet are even more distinctive for blood colouring matter than the bands of the visible spectrum. At a meeting of the International Physiological Congress at Berne in 1895 he described the following simple arrangement, which requires merely an electric arc lamp and an ordinary laboratory spectroscope of the Bunsen type, by which the bands in the ultra-violet may be rendered visible. "The telescope of the spectroscope is removed and a beam from the positive pole of the arc is allowed to fall on the slit of the collimator. The spectrum is focussed on a fluorescent screen, made by coating a white surface, such as cardboard, with barium platino-cyanide, and then the slit is opened very widely. If the spectrum be a continuous one (which is the case if it be that of the positive pole of the electric arc), the blood solution is then interposed in the path of a beam falling on the slit."

Improvements in the sensitising of photographic plates have enabled spectrograms to be made without the use of fluorescent screens. The following account is taken from Lewin, Miethe and Stenger (*Pflüg. Archiv.*, 1907, **118**, 82). For the blue and violet parts

of the spectrum alone an ordinary silver bromide gelatin plate suffices. In order to be able to photograph on one plate fine and coarse absorption bands in any part of the spectrum, the plate must be as far as possible equally sensitive to the whole spectrum, and no absorption bands, due to any material employed in making the plate, must be apparent. Such a continuous spectrum, extending from $\lambda = 375\mu\mu$ to $\lambda = 690\mu\mu$, is obtained either by the use of Wratten and Wainwright's panchromatic spectral plates or an isokol-bath plate.

Among coloured substances which can be used to sensitise a plate, *isokol*, (Bayer & Co., Elberfeld) is the best. The highly sensitive dry-plates prepared by Perutz are placed in the dark for 2 minutes with constant movement of the dish in an isokol-bath:

Isokol (1:1,000 alcohol)	5 c.c.
Alcohol	5 c.c.
Ammonia conc.	2 c.c.
Distilled water	200 c.c.

Franz, Rost and Heise use the "Agfa Plates" and place these for 4 minutes in

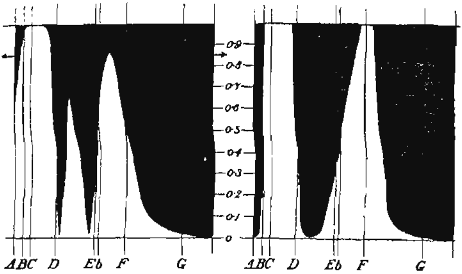
Isokol (1:1,000 of 90% alcohol)	5 c.c.
Alcohol, 90%	70 c.c.
Ammonia sp. gr. 0.960	2 c.c.
Distilled water	130 c.c.

The plates are washed in the dark in running water for 3 minutes, then placed in a perfectly dark drying chamber, through which dust-free air at 20° is aspirated. They should be dry in 20 to 30 minutes, as they are damaged by longer drying. The plates are put into the dark slide, and the development must commence in the dark, since the sensitiveness for red is so great. But after a minute the developing can be controlled with careful use of dark red light, since the plate, as it slowly develops, loses its marked sensitiveness to red light. For developing, 4 to 5% rodinol is used; it is then fixed in an acid bath. Instead of a prism, a diffraction grating is used, about 15,000 lines to an inch. As a source of light, a "Nernst" lamp or incandescent burner serves very well. The time of exposure depends on the kind of plate, the source of light, and the concentration of the solution investigated, and must obviously be determined by trial.

Spectrum of Oxyhæmoglobin.—The two characteristic bands, in the visible spectrum, are seen in greatest perfection in a stratum 1 cm

thick of a solution containing 1 per 1,000 of oxyhæmoglobin or 0.12–0.14% of blood. They are still perceptible in solutions of 1 in 100,000.

Fig. 6 illustrates the variations in the spectrum of the blood colouring matter corresponding to all concentrations, a stratum of 1 cm. being examined. In this diagram, the position of the Fraunhofer lines is shown; the numbers indicate percentage of oxyhæmoglobin, HbO_2, and reduced hæmoglobin. The shaded part



Oxyhæmoglobin. Hæmoglobin (reduced).

Fig. 6.—Graphic representation of the visible spectrum after A. Rollett. The figures represent percentages of the colouring matter.

indicates the absorption of light. By drawing lines parallel to the abscissæ we at once obtain the character of the absorption spectrum, corresponding to the concentration indicated by the vertical column of figures. The spectroscopic properties of the oxyhæmoglobin of all animals are the same, two absorption bands between D and E. With dilution the right-hand band disappears and only the left remains. According to Lewin, Miethe and Stenger, the mean of 112 measurements of different bloods gives maximal points for

$$\lambda = 577\mu\mu \text{ and } \lambda = 537\mu\mu$$

the oxyhæmoglobin of horse blood; the mean of 42 measurements was

$$\lambda = 579\mu\mu \text{ and } \lambda = 542\mu\mu$$

for the ultra-violet line (Soret's band); 50 determinations of rabbit's blood gave

$$\lambda = 415\mu\mu$$

and a similar value for the blood of man, pig and earthworm.

In the case of horse blood the mean of 42 measurements gave $\lambda = 413\mu\mu$. And for the mean of 134 measurements of pure oxyhæmoglobin, $\lambda = 415\mu\mu$. Lewin has examined blood 29 years old, which in solution was flocculent, brownish-black and showed no visible spectrum, and found the ultra-violet absorption, $\lambda = 408\mu\mu$. Another dried preparation of ox blood which was so insoluble in water that the extract in hot water showed no absorption even in a thick layer and gave no hemin crystals by Teichmann's method, still showed the ultra-violet band $\lambda = 415\mu\mu$. Also the yellow filtrate of blood or oxyhæmoglobin solutions which had been boiled repeatedly over a flame, still showed the violet absorption $\lambda = 415\mu\mu$.

In Fig. 7 we give the spectrum of oxyhæmoglobin (Rost, Franz and Heise). *A series of solutions of rabbit's blood of varying* concentration taken with a panchromatic plate by Wratten and Wainwright.

Spectrophotometric Properties of Oxyhæmoglobin.—The impression obtained by the unaided eye as to the intensity or breadth of an absorption band is often very fallacious. It is, therefore, necessary to determine quantitatively the amount of light actually absorbed by any medium, the absorption spectrum of which is being investigated; instead of trusting to the unaided sense of sight.

The qualitative estimation of hæmoglobin and its derivatives spectrophotometrically depends upon the determination of the ratio of the extinction coefficients in two definite parts of the spectrum of the colouring matter, on the assumption that this colouring matter undergoes no change during the experiment. This ratio is more or less characteristic for any given colouring matter, as Hüfner and his pupils have shown. If ϵ' is the extinction coefficient in one part of the spectrum and ϵ that in the other part of the same spectrum, the quotient ϵ'/ϵ for a definite derivate is constant, even for different concentrations and is different for different derivatives. If the molecular weights, of hæmoglobin and its derivatives were exactly known, the molecular extinction, that is to say, the extinction which 1 molecule or a definite fraction of 1 molecule dissolved in 1

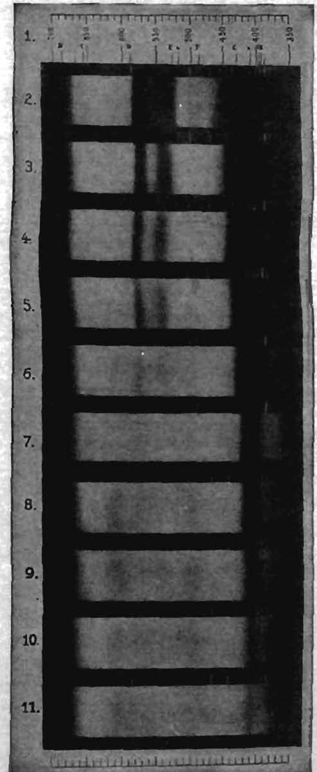


FIG. 7.—Spectrum of oxyhæmoglobin from Rost, Franz and Heise.

1. Scale of wave lengths.
2. Dilution, 1 : 70.
3. Dilution, 1 : 100.
4. Dilution, 1 : 150.
5. Dilution, 1 : 200.
6. Dilution, 1 : 300.
7. Dilution, 1 : 500.
8. Dilution, 1 : 800.
9. Dilution, 1 : 1,000.
10. Dilution, 1 : 1,500.
11. Dilution, 1 : 2,000.

litre of water exerts for a definite thickness of stratum in a definite region of the spectrum, would serve for its characterisation. Bunsen and Roscoe defined the extinction-coefficient as the reciprocal of that thickness of solution by which light of intensity J is reduced by $\frac{1}{10}$ in passing through the absorbing body. It is not necessary to know the absolute value of the intensity of the original light, since an absorbing body which weakens light of 1 by $\frac{1}{10}$ weakens light of intensity 2 by $\frac{2}{10}$, of 3 by $\frac{3}{10}$ . . . The coefficient of light diminution remains the same. Let ϵ be the extinction coefficient and m the thickness of solution by which light is reduced by $\frac{1}{10}$, then

$$\epsilon = \frac{1}{m}.$$

For the determination of the extinction coefficient we can also make use of the fact that the coefficient is, under the above assumption, a function of the thickness of the stratum of liquid, but with a constant thickness of stratum we can indirectly ascertain the extinction coefficient by measurement of the amount of light unabsorbed in the following manner.

Monochromatic light of intensity J passes through a stratum of coloured liquid of unit thickness, and is thereby weakened by J/n; in passing a second layer of the same thickness then according to Beer's law it suffers a further weakening of 1/n, so that the remaining light is now of intensity J/n^2. By passing through m such layers the residual light is now only J/n^m. In other words, as the thickness of the stratum of liquid increases in arithmetical progression, the intensity of light decreases in geometrical progression.

Let the original intensity of light J = 1, and intensity of the light after passing through m strata, J′, then

$$J' = \frac{1}{n^m} \text{ or } n^m = \frac{1}{J'} \qquad (1)$$

Taking logarithms and transposing, we get

$$\log n = \frac{-\log J'}{m} \qquad (2)$$

From the definition of the extinction coefficient, it follows that $m = 1/\epsilon$ since $J' = \frac{1}{10}$, and substituting these values in equation (2); we obtain

$$\log n = \epsilon$$

and, therefore, also

$$\epsilon = -\log \frac{J'}{m}$$

If we choose the thickness of m as 1 cm,

$$\epsilon = -\log J',$$

that is to say, the extinction coefficient in this case is equal to the negative logarithm of the intensity of the residual light.

If one determines with the spectrophotometer the intensity of the residual light after absorption by a stratum of hæmoglobin or one of its derivatives, a simple calculation is only necessary for ascertaining *the extinction coefficient. But for such measurement it presupposes* that light as monochromatic as possible is used.

The Spectrophotometer of Hüfner.—In this instrument many of the inconveniences and sources of error in the original form employed by Vierordt are eliminated. (For description of the form supplied by Hilger, see Vol. I, page 39.) In this instrument light, polarised by a Nicol, falls on one half of the slit of a spectroscope, while unpolarised light falls on the other half. The slit should not be open more than 0.5 mm. The unequal brightness of the two beams is compensated by a smoked glass wedge. After the brightness of the two beams has been rendered unequal by the interposition of the absorbing liquid, the second Nicol in the telescope is rotated until the two fields appear equally bright. The angle of rotation φ is measured, and the extinction coefficient found as follows. If J° is the intensity *of the polarised ray before rotation and J′ the intensity after rotation, then*

$$J^{\circ} - J' = \sin^2 \varphi$$

and if $J^{\circ} = 1$, then $J' = -\cos^2 \varphi$, and therefore the extinction coefficient $\epsilon = -\log J' = -\log \cos^2 \varphi = -2 \log \cos \varphi$.

The hæmoglobin solution is placed in a glass cell with parallel walls 11 mm. apart; in the lower half of this cell is a glass block known as "der Schulz'sche Glaskörper" exactly 10 mm. broad and half the height of the interior of the cell. It rests upon the floor of the cell, so that the anterior and posterior surfaces of the blood shall be parallel to the glass plates of the trough. When light traverses the lower half it passes through a stratum 1 mm. in thickness; when the upper half, 11 mm. in thickness.

TABLE 3

Colouring matter	Wave-length of spectral region in μμ For ε′ 531.5–542.5 (a^1) or 534.0–542.0 (b^1) For ε 534.0–565.0 (a) or 556.5–564.5 (b)	Quotient	Author
HbO_2[1]	a^1 or b^1 a or b	$\frac{\epsilon'}{\epsilon} = 1.578$	Hüfner, *Arch. Physiol.*, 1894, 130.
Met-Hb alkaline	a^1 and a	$\frac{\epsilon'}{\epsilon} = 1.19$	Zeynek, *Arch. Physiol.*, 1899, p. 460.
Met-Hb neutral	b^1 and b	$\frac{\epsilon'}{\epsilon} = 1.58$	Bürker. *Tigerstedt's Handb. f. Physiol. Methode*, 1910.
Hb-CO	a^1 and a	$\frac{\epsilon'}{\epsilon} = 1.10$	Hüfner, *Arch. Physiol.*, 142.
Hb-NO	D63E-D84E and D32E-D53E	$\frac{\epsilon'}{\epsilon} = 1.05$	Hüfner and Kulz, *Z. physiol. Chem.*, 1883, 7, 366.
Hb-CN	a^1 and a	$\frac{\epsilon'}{\epsilon} = 1.21$	Zeynek, *Z. physiol. Chem.*, 1901, **33**, 426.
Hb (reduced)	a^1 and a	$\frac{\epsilon'}{\epsilon} = 0.76$	Hüfner, *Arch. physiol.*, 1894, 130.
Hæmatin alkaline	b^1 and b	$\frac{\epsilon'}{\epsilon} = 1.09$	Bürker, *ibid.*
Hæmatin acid	b^1 and b	$\frac{\epsilon'}{\epsilon} = 1.25$	*Ibid.*
Hæmochromogen	b^1 and 534–562	$\frac{\epsilon'}{\epsilon} = 0.50$	*Ibid.*
Hæmatoporphyrin alkaline	b^1 and b	$\frac{\epsilon'}{\epsilon} = 1.11$	*Ibid.*
Hæmatoporphyrin acid	b^1 and b	$\frac{\epsilon'}{\epsilon} = 0.76$	*Ibid.*

[1] *Hb = Hæmoglobin.*

The spectrophotometer constants of oxyhæmoglobin are given in Table III, taken from K. Bürker, along with the constants of the other derivatives. The determination of these is an essential method for the accurate qualitative recognition of hæmoglobin and its derivatives. Butterfield (*Z. physiol. Chem.*, 1909, **62**, 178–204) has shown that the constancy of the quotient ϵ'/ϵ holds not only for fresh normal blood of men and animals but for human blood in

pathological states, such as polycythæmia, chlorosis, pernicious anæmia and scurvy.

These figures have been repeatedly confirmed, most recently by Hari (*Biochem: Z.*, 1917, **82**, 229), who also gives a full account of the literature. He finds that solutions give at the beginning, during the first 15 minutes after solution, a higher value—about 1.60.

The Heat of Combination of Hæmoglobin and Oxygen.—According to Barcroft and A. V. Hill (*J. Physiol.*, 1910, **39**, 427) the mean value for 1 grm. of hæmoglobin is 1.85 calories. According to Berthelot, the value is 14.77 calories. Torup's value is similar.

Chemical Properties.—Hæmoglobin dissolved in water or, better, in 0.1% sodium hydroxide solution, shaken with air unites with a definite quantity of oxygen. At 0° C. and 760 mm. 1 grm. of hæmoglobin unites witb 0.001915 grm. = 1.34 c.c. of oxygen. Oxyhæmoglobin is possibly a peroxide, $\mathrm{Hb}\!<\!\begin{array}{c}\mathrm{O}\\ |\\ \mathrm{O}\end{array}$, and is a dissociable compound, so that diminution of the oxygen tension in the atmosphere to which oxyhæmoglobin is exposed results in a certain degree of dissociation. The oxygen tension can reach zero by passage of indifferent gases, such as hydrogen or nitrogen, by exposure to a vacuum, or by reducing agents. Various curves, indicating the grade of dissociation of oxyhæmoglobin, or what is the same, the extent of saturation of hæmoglobin with varying pressures of oxygen, have been published by Hüfner (*Arch. Physiol.*, 1901, Suppl. Bd., 187), Krogh (*Skand. Archiv.* 1903, **16**, 400), Bohr (*Skand. Archiv. Physiol.*, **16**, 409) and Barcroft and Roberts (*J. Physiol.*, 1910, **39**, 118). The dissociation curves of the blood of different animals show some difference, attributable to difference in the saline constituents of the red corpuscles. A clear discussion of the evidence for and against the chemical nature of specific oxygen capacity in hæmoglobin was published by Peters (*J. Physiol.*, 1912, **44**, 131).

Oxyhæmoglobin has the character of a weak acid and apparently forms with correspondingly weak alkaline solutions a salt; with stronger alkali it is decomposed.

Blood colouring matter, especially in the form of oxyhæmoglobin, acts as a *catalyst*, decomposing hydrogen peroxide into water and

oxygen; and the purer the hæmoglobin the less active is it in this respect. In connection with this property, a number of chemical tests for blood have been devised. These are of some considerable diagnostic value, and are given by hæmoglobin and its compounds and all its iron-containing hydrolytic derivatives. Substances such as hæmatoporphyrin, hæmatoidin or bilirubin, which are iron-free derivatives, do not give the tests now to be described.

1. **The guaiacum reaction** of Schönbein first described in 1858. In later communications (1863) Schönbein directed attention to the delicacy of the guaiacum-peroxide test for blood, and to its value in forensic investigations. A sapphire-blue colour (guaiacum blue) develops when blood, hydrogen peroxide, and alcoholic solution of guaiacum are mixed in a test-tube. The guaiacum test is also known as Van Deen's, Almen's, or Vitali's test. Instead of hydrogen peroxide, ozonised ether or ozonised oil of turpentine have been employed; and instead of alcoholic solution of guaiacum resin, an alcoholic solution of guaiaconic acid, $C_{20}H_{24}O_6$, is to be preferred. (Van Deen, *Arch. f. d. holländ. Beitr. z. Natur. u. Heilkunde*, Utrecht, 1861–1864, **3**, *228*; Taylor, Guy's Hosp. Reports, 1868, **13**, 431; 1870, **15**, 273; 1874, **19**, 517; Buckmaster, *J. Physiol.*, 1897, 35; Carlson, *Z. physiol. Chem.*, 1906, **48**, 69, and 1908, **55**, 260; Whitney, *Brit. Med. and Surg. J.*, 1909, **160**, 202. Lesser, *Z. f. Biol*, 1907, **49**, 571; Liebermann, *Pflüger's Arch.*, 1904, 227. See also J. H. Kastle, Chemical Tests for Blood, *Hygienic Lab. Reports of the Public Health and Marine Hospital Service*, 1909, Bull. 51.

The general theory of the reaction is a matter of uncertainty. The presence of iron in the blood pigment is essential, and the test must be made with some care and with controls. Blood exposed to a temperature of 200° still gives the test. If the material to be tested (blood) is left in contact with even weak hydrogen peroxide solution and then, 5 minutes later, guaiaconic acid is added, the reaction fails; the products of the chemical change, therefore, do not blue guaiacum.

The guaiacum-peroxide test for blood is not due to any enzyme. It resembles, but is in its nature quite dissimilar from any of the peroxydase reactions hitherto described. For convenience, the reaction with blood may be termed a pseudo-peroxydase reaction.

The test is carried out as follows:

A 1% solution of guaiaconic acid (Merck) in equal parts of alcohol and water is freshly prepared. A few c.c. are placed in a test-tube,

then half this volume of 3% hydrogen peroxide. On adding the blood solution, the guaiacum blue colour develops, and slowly disappears in about 30 minutes. O. Schumm regards ozonised turpentine oil as a better reagent than peroxide. For this method of preparing an active ozonised oil, see *Z. physiol. Chem.*, 1906, **50**, 374. Bürker, Carlson, and Buckmaster consider that weak peroxide solutions are preferable.

Delicacy of the guaiacum test for detection of blood and water:

1–5,000 of water, Adler, *Z. physiol. Chem.*, 1904, **41**, 59.

1–40,000 of water, Limon, *Vrtlijhrsch. f. ger. u. öff. Med.*, 1863, **24**, 193.

1–40,000 to 100,000, Schumm, *Arch. Pharm.*, 1907, **247**, 1.

1–20,000 to 40,000 (urine) Schumm, *Arch. Pharm.*, 1907, **247**, 1.

In the writers' experience the guaiacum test is of especial value in the detection of blood. It is given by blood-solutions which have been boiled, and this should be done before carrying out the test, so as to avoid any possibility of the colour being produced by ferments. The test is more sensitive with great dilutions of laked blood compared with unlaked blood. The test can be obtained with dilutions of 1 in 100,000.

The Benzidine Test of O. Adler and R. Adler (*Z. physiol. Chem.*, 1904, **41**, 76).—Benzidine or paradiamino-diphenyl, $NH_2C_6H_4C_6H_4NH_2$, preparations are not all of equal value for this test (O. Schumm and C. Westphal, *Z. physiol. Chem.*, 1905, **46**, 510). The solution should be fresh. A few mg. of Merck's benzidine are dissolved in 2 c.c. of glacial acetic acid. 10 drops are placed in a clean test-tube, and then 30 c.c. of 3% hydrogen peroxide added, when no colour should appear. The blood or suspected fluid is then added, and the development of a blue-green coloration indicates the presence of blood. Einhorn (*Deutsche med. Woch.*, 1907, 1089) soaks white filter paper with the acid solution and dries, then dips a strip in the fluid to be tested, and drops peroxide solution on this, or the paper may be laid in the solution.

Hydrogen peroxide for these tests may be conveniently made by dissolving pure sodium perborate in acetic acid. According to A. Lucas a sensitive solution of benzidine may be made as follows:

0.2 grm. of dry sodium perborate is dissolved in 1 c.c. of water and 10 c.c. of glacial acetic acid. To this 1 c.c. of a 10% solution of benzidine in glacial acetic acid is added. If sodium perborate is

not available, 2 c.c. of 6% hydrogen peroxide (free from acid) are substituted. *It should be prepared just before use, as it keeps only* a few hours. The test is carried out as follows:

A double layer of good filter paper is spread on a porcelain tile, and a minute fragment of dried blood or blood-stained fabric is laid upon it. A drop of benzidine solution is added by means of a pipette. A dense blue coloration, radiating out in streaks on the filter paper is produced *instantaneously* if blood is present. Moistening the material with pyridine before adding the benzidine solution promotes rapidity of action.

The reaction is a delicate one; blood can be detected in a dilution of 1:100,000 (Adler) or 1:200,000 (Schumm), 1:300,000 (Ascarelli), when using Einhorn modification in 1:80,000. With two of Kahlbaum's preparations the limit was 1 part to 2,000,000; with some others of the same firm 1:50,000 (Schumm).

The Aloin Test of Rossel (*Deutsche Archiv klin. Med.*, 1903, **76**, 505). Solutions of aloin (Merck's preparation from Curaçoa aloes) react exactly like solutions of guaiaconic acid to hæmoglobin, its derivatives and hæmatoporphyrin. The aloin solution must always be freshly prepared; 0.25 grm. to 50 c.c. of equal parts of water and alcohol. The clear yellow liquid is the test solution. 10–20 drops of this in a test-tube are mixed with twice its volume of 3% hydrogen peroxide. There is no change in colour. The suspected blood is *then added, and a purple-red colour gradually develops.* The colour is much more stable than that of guaiacum blue, but the aloin reaction is less sensitive. (Schaer, *Arch. Pharm.*, 1900, **238**, 42.)

The Malachite Green Reaction of O. Adler and R. Adler (*Z. physiol. Chem.*, 1904, **41**, 59).—The leuco-base of malachite green (tetramethyldiaminotriphenylmethane) is a white crystallin substance insoluble in water, but soluble in weak acids. Such solutions are easily oxidised to a peacock blue colour (malachite green). Adequately diluted solutions give a distinctive absorption spectrum, and the centre of the band nearly coincides with the sodium line *D*. From this property, as v. Czyhlarz and v. Fürth have shown, it is possible to measure with accuracy the rate at which the leuco-base is oxidised by hæmin in the presence of hydrogen peroxide. (*Hofmeister's Beitr.*, 1907, **10**, 363; Buckmaster, *J. Physiol.*, 1908, 37.)

A solution of 0.25 grm. of leuco-base in 163 c.c. of water and 12 c.c. of glacial acetic is prepared. 10 drops of this solution and three

times the quantity of 3% peroxide solution are placed in a perfectly clean test-tube. Then the blood is added. This should be boiled before the test is made.

The test solution slowly turns green when kept in a stoppered bottle. This can be obviated to some extent by saturating the liquid with carbon dioxide and keeping in an atmosphere of this gas. The reaction is exceedingly delicate, at least as sensitive, or more so than the guaiacum test; 1 part of blood in 100,000 of water can easily be detected. The leuco-base, unlike guaiaconic acid, is not oxidised by 3% hydrogen peroxide. Buckmaster has shown, (1) that the extent to which leuco-base is converted into malachite green depends entirely on the quantity of blood-pigment present; (2) that the reaction, occurring with boiled blood pigment, is not due to a ferment; (3) that removal of the iron from hæmoglobin, forming hæmatotoporphyrin or *hæmatoidin, abolishes the reaction.*

The **paraphenylenediamine hydrochloride,** ($C_6H_4(NH_2)_2$, 2HCl), test of J. Boas (*Centralbl. f. inner. Med.*, 1906, **27,** 601): 0.5% aqueous solution of this substance is used. 1–2 drops of reagent placed in a test-tube (with the addition, if acid, of sufficient $N/2$ alcoholic potash to neutralise this) then 15–20 drops of 3% hydrogen peroxide, and finally the solution to be tested. If hæmoglobin or any of its iron-containing derivatives are present, an olive-green colour develops.

Schumm and Remstedt advise the addition of 1–5 drops of weak acetic acid to the mixture. The olive-green colour gradually passes into a brown-red.

The test is not so delicate as the guaiacum or benzidine reaction.

Phenolphthalin Test for Blood.—Utz (*Chem. Ztg.*, 1907, **31,** 737) regards phenolphthalin as a more delicate test for blood than either guaiacum or aloin. Blood at a dilution of 1:1,000,000 could be detected (Delearde and A. Benoit, *Compt. rend.*, 1908, **64,** 960–992). J. H. Kastle (*Ibid.*, p. 30), using a Duboscq colorimeter, states that he recognised blood at a dilution of 1:80,000,000.

Phenolphthalin is the leuco-compound of phenolphthalein. It is colourless in alkaline solutions, but on adding alkali to the substance after oxidation, or if it be oxidised in the presence of alkali, the purple-red colour of phenolphthalein in alkali is produced. (For the preparation of phenolphthalin see Kastle.) The reagent is prepared by dissolving 0.032 grm. phenolphthalin in 21 c.c. $N/10$ sodium hydrox-

ide, and making up to 100 c.c. with pure redistilled water. To this 0.1 c.c. of $N/1$ hydrogen peroxide is added. The purest sodium hydroxide must be used, and redistilled water for all dilutions. On the addition of diluted blood the colour develops at room temperature in 5 minutes. When an alkaline solution of phenolphthalin without peroxide is used the colour develops in 18 hours at room temperature. 1 part of blood in 8,000,000 could be detected. Moreover, the quantity of phenolphthalin oxidised under the *influence of blood is proportional to the quantity of blood present.*

The test is interfered with, or retarded by, substances present in tissues, such as liver, spleen, pancreas or urine. In the latter fluid about 1 part of blood in 390,000 of urine could be detected.

The phenolphthalin test for blood is exceedingly delicate, but copper salts, chrome alum, mercuric chloride, lead chloride and potassium ferricyanide all show, in some degree, a similar oxidation of this reagent.

The chief objection urged against the guaiacum test for blood (most observers considering that its value lies in indicating the absence of blood when the reaction is negative) is that a large number of substances other than blood give a positive reaction, the following among others: all oxidising agents, the halogens, finely divided metals such as copper, iron, and compounds of cobalt, chromium, copper, gold, iron, manganese and lead. Also hypochlorites, iodides, bromides, nitrates, nitrites, and sodium chloride. Milk, pus, saliva, fæces and sweat, together with many substances of vegetable origin derived from asparagus, potatoes, flour, tomatoes, oats, peas and meal may also give the guaiacum reaction.

The general consensus of opinion among those who have worked at this reaction is that the guaiacum test, carried out as above directed, is a valuable one If there is no reaction, then the absence of blood can be inferred. If a positive reaction occurs, care should be taken to exclude any enzymic action due to oxidases or peroxidases, by boiling for some minutes, and then subjecting the material to confirmative tests.

Hæmoglobin (Hb, reduced hæmoglobin, or purple cruorin).—In 1864 Stokes, in a communication to the Royal Society, showed that when blood is treated with certain reducing agents the colour of the liquid and the spectrum undergo remarkable changes, and concluded from his experiments "that the colouring matter of blood, like

indigo, is capable of existing in the state of oxidation, distinguishable by a difference of colour and a fundamental difference in the action on the spectrum. It may be made to pass from the more to the less oxidised state by the action of suitable reducing agents, and recovers its oxygen by absorption from the air."

Methods of Reducing Oxyhæmoglobin to Hæmoglobin.—In all these experiments diluted blood may be substituted for solutions of the pure colouring matter. Instead of very pure water it is advisable to use 0.1% solution of sodium hydroxide. It is essential that the reducing agent should not act destructively on the hæmoglobin. The reduction of oxyhæmoglobin seldom proceeds quantitatively (Hüfner). Generally, secondary reaction takes place to some degree, whereby part of the loosely combined oxygen is liberated and oxidises not only other substances but the molecule of colouring matter itself.

1. *Stokes' Reagent.*—(Alkaline solutions of ferrous salts.) To a solution of ferrous ammonium sulphate, tartaric or citric acid, or one of the alkaline salts of these acids is added, and then ammonia until the reaction is just alkaline. The light green solution obtained reduces oxyhæmoglobin rapidly, even in the cold. This solution presents the disadvantage that it itself becomes oxidised and therefore coloured, and absorbs the more refrangible rays of the spectrum and interferes with the exact study of the absorption bands. To get over this difficulty, Stokes subsequently substituted stannous chloride for a ferrous salt.

2. *Alkaline sulphides* also effect the reduction, but more slowly than Stokes' reagent. Ammonium sulphide is a good reagent, and is best prepared by saturating strong ammonia with hydrogen sulphide and then adding an equal volume of strong ammonia. The solution must be freshly prepared and colourless, since if yellow it absorbs the violet end of the spectrum. Ordinary solutions of ammonium sulphide should not be used, as they lead to the formation of sulph-methæmoglobin. Many workers prefer to use a freshly prepared solution of 40% potash saturated with hydrogen sulphide. The solution should be clear and colourless.

3. Ludwig and Schmidt used *ferrum redactum.*

4. *Sodium hydrosulphite*, $Na_2S_2O_4$, has intense reducing power. 2 grm. of sodium hydrosulphite are shaken up in a stoppered test tube with 5 c.c. of 10% potassium hydroxide solution and 1 c.c. of

alcohol. The solution is best kept in a stoppered bottle and must be prepared just before use. It possesses the advantage of not greatly increasing the volume of the blood solution, and its solution is also clear and colourless.

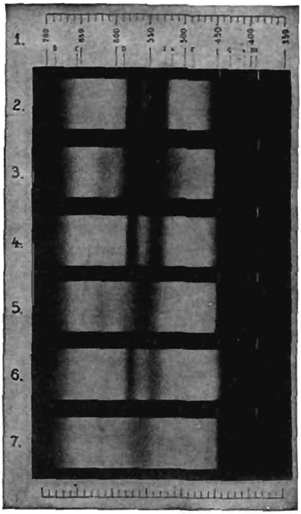


FIG. 8.—(From Franz, Rost and Heise.)
1. Scale of wave lengths.
2. *Normal rabbit's blood*, *1 : 170*.
3. Normal rabbit's blood reduced by ammonium sulphide.
4. Normal rabbit's blood, 1 : 100.
5. Normal rabbit's blood reduced by ammonium sulphide.
6. Normal rabbit's blood, 1 : 150.
7. Normal rabbit's blood reduced with ammonium sulphide.

5. *Hydrazine*.—The 50% solution of hydrazine hydrate of commerce easily reduces oxyhæmoglobin, and has the advantage that the only products of its decomposition are nitrogen and water, $NH_2NH_2,H_2O + O_2 = N_2 + 3H_2O$. If we know the quantity of

oxyhæmoglobin to be reduced, the calculated quantity of hydrazine hydrate can be added. Riegler uses a solution of hydrazine made by dissolving 5 grm. of sodium hydroxide and 2.5 grm. of hydrazine sulphate in 50 c.c. of water and adding 50 c.c. of 96% alcohol. After standing a few hours the mixture is filtered. It must not be used in excess, otherwise the hæmoglobin is decomposed into hæmochromogen and globin.

6. *Auto-reduction by Bacteria.*—The solutions are sealed up in tubes and kept at 40°, when reduction rapidly takes place.

7. By removal of the oxygen of oxyhæmoglobin in a Torricellian vacuum.

8. By the passage of such gases as nitrogen or hydrogen through solutions of oxyhæmoglobin. The hydrogen must be purified by passing through concentrated potassium permanganate and then concentrated potassium or sodium hydroxide.

The crystals of hæmoglobin were prepared by Kühne by placing a concentrated solution of pure oxyhæmoglobin in very dilute ammonia in a gas chamber and passing pure dry hydrogen over it; as the solution evaporated the crystals separated as hæmoglobin. Gamgee placed a magma of pure hæmoglobin crystals, together with some of the mother-liquor from which they had separated, in a glass tube and then sealed it off just above the surface of the liquid. He placed this in an incubator at 35° for some days, then set it aside in an ice chamber, and after many weeks the tube was found to contain crystallised and perfectly reduced hæmoglobin. The crystals are doubly refracting, pleochromatic, and practically identical in form with those of oxyhæmoglobin.

In Fig. 8, we give comparative spectra of oxy- and reduced hæmoglobin.

In thick layers, solutions of hæmoglobin are dark claret-coloured; in dilute solutions greenish. This dichroism is characteristic of the blood of asphyxiated animals.

Spectrum.—When oxyhæmoglobin is reduced, the two absorption bands disappear, and are replaced by a single band between D and E, which is less deeply shaded and possesses less sharply defined edges. (The right hand diagram of Fig. 6 shows the various degrees of absorption of light by various strengths of solution of hæmoglobin.) Lewin, Miethe and Stenger give the point of maximum absorption as $\lambda = 558\mu\mu$ for crystallised hæmoglobin, prepared from oxyhæmoglobin and $\lambda = 559\mu\mu$ for reduced hæmoglobin from blood.

The photographic spectrum, taken with a perortho plate gives a band $\lambda = 429\mu\mu$, so that the band characteristic of oxyhæmoglobin is displaced toward the red end of the spectrum by $14\mu\mu$. For the spectrophotometric constants see Table 3.

Carbon-monoxide Hæmoglobin (Carboxyhæmoglobin).—When a current of carbon monoxide is led through a solution of oxyhæmoglobin or blood, the solution acquires a cherry-red colour, in contrast to the scarlet of oxyhæmoglobin. If the original solution was sufficiently concentrated, then on cooling to 0°, and adding a quarter of its volume of alcohol also at 0°, and setting aside to cool in a chamber below 0°, after some hours or days crystals of CO-Hb separate. They are identical in form with oxyhæmoglobin, but are less soluble. Hüfner has shown that exactly the same volume of carbon monoxide combines with hæmoglobin as oxygen, 1 grm. of hæmoglobin uniting with 1.338 c.c. of CO at 0° and 760.

Carboxyhæmoglobin is more stable than oxyhæmoglobin. The affinity of hæmoglobin for carbon monoxide is 154 times that which it possesses for oxygen (Haldane, *J. Physiol.*, 1895, **18**, 430). Solutions of carboxyhæmoglobin yield up carbon monoxide to the vacuum of the blood pump, but less readily than oxyhæmoglobin; and if present in small quantity carbon monoxide is difficult to recover by this method unless the blood is acidified. The continued bubbling of indifferent gases through solutions of CO-hæmoglobin results in finally driving out the carbon monoxide, a solution of hæmoglobin remaining. (Gamgee, *J. Anat. Physiol.*, 1867, **1**, 339.) The gas can also be extracted from carboxyhæmoglobin by treatment with a strong solution of potassium ferricyanide. The solutions are cherry-red in tint, and in great dilution typically pink. If concentrations of equal strength of oxy- and carboxyhæmoglobin are compared by means of the spectroscope, it will be found that the CO compound absorbs the blue rays of the spectrum to a less degree than the oxyhæmoglobin.

Spectrum.—Carboxyhæmoglobin shows two bands of almost equal intensity and breadth in the yellow-green part of the spectrum. A band can also be detected in the violet. As a mean of 60 measurements of the spectrograms, Lewin, Miethe and Stenger give as the points of maximal absorption $\lambda = 570\mu\mu$, $\lambda = 542\mu\mu$, $\lambda = 416\mu\mu$.

The position of these bands is not altered by the addition of reducing reagents. The spectrophotometric constants are given in the table 3, (p. 29).

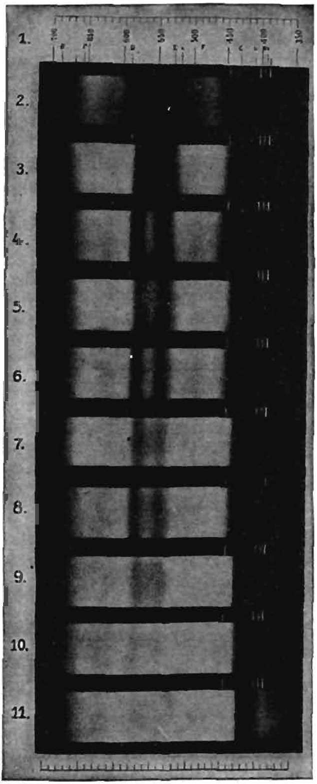


FIG. 9.

1. Scale of wave-lengths.
2. HbO_2. 1 : 70.
3. HbCO. 1 : 70.
4. HbO_2. 1 : 100.
5. HbCO. 1 : 100.
6. HbO_2. 1 : 150.
7. HbCO. 1 : 150.
8. HbO_2. 1 : 200.
9. HbCO. 1 : 200.
10. HbO_2. 1 : 500.
11. HbCO. 1 : 500.

In Fig. 9 we give the spectrum of carboxy- and oxyhæmoglobin, taken from Rost, Franz, and Heise, rabbit's blood being diluted and then converted into carboxyhæmoglobin with carbon-monoxide gas.

The addition of a concentrated solution of sodium hydroxide (sp. gr. 1.3) to a solution of blood saturated with carbon monoxide in the proportion of 10 to 1, causes the blood to assume a fine cinnabar red colour and deposit a cinnabar red precipitate. The same precipitate is produced with solutions of pure carboxyhæmoglobin. Normal blood or oxyhæmoglobin solutions treated in the same way gives a shiny black mass, which is greenish-brown when spread out on a porcelain slab.

According to Kunkel, tannic acid is useful for the detection of Hb-CO. With diluted normal blood this gives a greenish-brown, with Hb-CO a bright carmine-red precipitate.

Pure aqueous solutions of carboxyhæmoglobin, when boiled, give a bright red precipitate, composed, according to Hoppe-Seyler, of coagulated protein and CO-hæmochromogen. Solutions of carboxyhæmoglobin exposed to nitric oxide in the absence of oxygen, are at once decomposed. Nitric oxide hæmoglobin is formed and carbon monoxide liberated.

The use of the Hartridge reversion spectroscope for the detection of carbon monoxide and the determination of the percentage saturation of blood was dealt with by R. C. Frederick in a communication to the Society of Public Analysis (*Analyst*, 1931 **56**, 561).

Apart from its behaviour to hæmoglobin, carbon monoxide is a perfectly indifferent gas for respiration. Since the relative affinities of carbon monoxide and oxygen for hæmoglobin are as 154:1, in an atmosphere of 1 volume CO and 154 volumes O_2 the hæmoglobin present in blood takes up equal quantities, and the hæmoglobin is half saturated with O_2, half with CO. If the normal alveolar air of the lungs contains 15% of oxygen, then 0.1% carbon monoxide in the respired air will half saturate the available hæmoglobin; with 0.3% of carbon monoxide the saturation of blood with this gas would be three-quarters.

In any sample of blood which is suspected to contain carboxyhæmoglobin this can be estimated by Haldane's carmine method.[1] As it is known that oxyhæmoglobin sufficiently diluted is yellow,

[1] Carmine solutions: 1 grm. pure carmine is mixed in a mortar with a few drops of ammonia and dissolved in 100 c.c. of glycerin. The standard solution is made fresh by adding 5 c.c. to 500 c.c. of water.

while carboxyhæmoglobin is pink, this latter tint can be easily matched with carmine solution.

Narrow test-tubes, about 0.5–0.75 cm. in diameter, are used. In each of three of these, A, B, C, 2 c.c. of water are placed. 20 c.c. of the blood to be examined are mixed with water in A. Normal blood similarily diluted is placed in B and also in C. Tube B is then saturated with carbon-monoxide gas, so that all the blood-colouring matter is in the state of carboxyhæmoglobin. To tube C (normal diluted blood) standard carmine is added, 0.1 c.c. at a time, until it equals the tint of A; then more, until it equals the tint of B. Then the amount of carboxyhæmoglobin in tube A can be calculated. Suppose 0.5 c.c. of standard carmine must be added to C to make its tint equal to A, and 2.2. c.c. of standard carmine to produce equality of tint with B, then since 2 c.c. of water is in each tube, $A = \frac{0.5}{2.5} \times \frac{4.2}{2.2} \times 100 = 38\%$ saturation or of 100 parts of hæmoglobin 38 parts are carboxyhæmoglobin and 62 parts oxyhæmoglobin.

Carboxyhæmoglobin can be dissociated by means of potassium ferricyanide in the same manner as oxyhæmoglobin (Haldane).

For obtaining the gases,—oxygen or carbon monoxide, about 20 c.c. of blood are mixed with 30 c.c. of $\frac{1}{500}$ ammonia. The blood is thereby laked and 4 c.c. of a saturated solution of potassium ferricyanide is mixed with this. All the associated oxygen or carbon monoxide is evolved and collected over water. The amount of gas so liberated is equal to that obtained by exposure of the blood at a temperature of 40° to a Torricellian vacuum. This experiment can be carried out with the ordinary Dupré's apparatus for determination of urea or with the original apparatus (Fig. 10) used by Haldane. For small quantities of blood the apparatus of Barcroft and Haldane can be employed (Barcroft and Roberts, *J. Physiol.*, 1909, **39**, 429).

Buckmaster and Gardner (*Proc. Roy. Soc.*, 1909, **B. 81**, 575) found that minute quantities of carbon monoxide in the blood, too small to be recognised by other methods, can be detected as follows: The suspected blood is treated with potassium ferricyanide, as described above, and the gases which are evolved are allowed to escape into a closed space containing about 50 c.c. of air. The form of apparatus is a small bottle in which the blood and ferricyanide are mixed, connected with a Hempel gas-burette fitted with a 3-way tap. The

gas mixture is displaced into the burette and then bubbled very slowly through a solution of blood and distilled water in a narrow test-tube. The solution is so dilute that in looking down the tube it appears a reddish-yellow tint. The tint of this is then compared with a control sample of the same diluted blood solution. For comparisons simple dilutions are made, containing the same quantity of colouring matter as the control sample, and the sample through

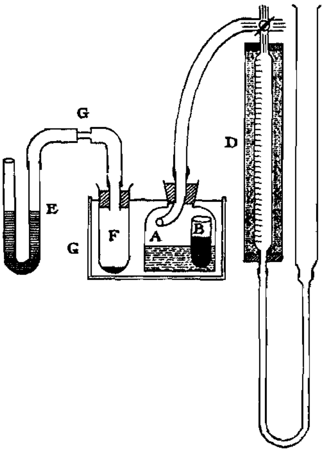


FIG. 10.—A is a corked bottle holding blood and ammonia; B, a test-tube holding ferricyanide of potassium prior to mixing. C is a water-bath; D, gas burette and levelling tube; E, a water-gauge attached to the temperature and pressure control tube F, which is adjusted by sliding the rubber tubing backward or forward on the glass tube G.

which the gas has been bubbled. The latter tube shows the characteristic bluish-pink tint of carboxyhæmoglobin. The two tubes are examined side by side, or in a tintometer by looking through the column of liquid against a white surface. An even more delicate way of detecting any difference in the two samples is to fill 20 cm. polarimeter tubes, placed side by side in a dark box and viewed through a ground glass screen suitably illuminated with blue light.

In this way minute quantities of carboxyhæmoglobin can be detected. For instance, 0.5 c.c. of blood from a cat was saturated with carbon monoxide and reintroduced into the blood stream of the animal. *Half an hour later the animal was killed, and by the method just* described carboxyhæmoglobin recognised with certainty in the blood. (The blood volume of a cat weighing 3 kg. is about 100 c.c., so that the hæmoglobin was not more than 1/200 saturated.)

Also carbon monoxide was easily recognised by this method in 5 c.c. of air containing 0.8% of the gas and quite readily in 10 c.c. of air containing 0.2% of carbon monoxide.

A. P. Fokker (*Chem. Centralb.*, 20, 380; *J. Soc. Chem. Ind.*, 1884, p. 579) has described the following process, by which it is claimed that it is possible to detect carbonic oxide in a single drop of blood. From 1 to 2 c.c. of the blood to be examined is placed in a small deep beaker, clamped between three bent brass wires. The upper ends of these wires support a watch-glass containing a solution of palladious chloride, while the lower ends are soldered to a round brass plate. The arrangement is placed in a porcelain vessel filled with water, and covered with a narrow bell-glass. About two-thirds of the water is then sucked out of the bell-jar by means of an india-rubber tube. The water rises in the bell-jar, and the beaker, kept upright by the brass plate, swims on its surface. The water is then heated to boiling, when the blood coagulates, and the palladious chloride is reduced by the liberated carbonic oxide. If only traces of this gas are present, the reduction is not complete, and the arrangement must be allowed to stand for 24 hours. Free ammonia, if present, will produce a yellow amorphous compound with the palladium chloride, but carbonic oxide forms a brilliant black metallic mirror. A similar reaction is produced by hydrogen sulphide, which is, however, rarely present in fresh blood.

Desgrez and Nicloux stated, as the result of experiments carried out on dogs in Paris, that carbon monoxide is not only a normal constituent of the blood-gases, but that the blood of these animals once anæsthetised by chloroform contains an augmented quantity of the gas. (*Arch. Physiol.*, 1898, 2, 377) Nicloux also states that when blood is incubated *in vitro* with chloroform, carbon monoxide is produced. The method these observers used for the detection of carbon monoxide was to pass the evacuated blood-gases over solid potassium hydroxide to absorb the carbon dioxide and then over

iodine pentoxide at 150°. The carbon monoxide decomposes iodine pentoxide according to the following equation:—

$$I_2O_5 + 5CO = I_2 + 5CO_2.$$

The iodine liberated is collected in a suitable solvent and estimated. The writers have been unable to confirm the existence of carbon monoxide either in the blood of cats in London, or during chloroform narcosis, or by incubating chloroform and blood together, and their experiments show that the carbon monoxide found by Nicloux in the blood of chloroformed animals is due to the carbon monoxide produced when the chloroform vapour which is contained in the blood gases is passed over potassium hydroxide to absorb the carbon dioxide. Chloroform vapour is very readily decomposed either by solutions of potassium hydroxide or by solid potassium hydroxide yielding carbon monoxide;

$$CHCl_3 + 3KOH = 3KCl + CO + H_2O.$$

The small quantities of carbon monoxide found in the blood of normal animals is probably due to the fact that iodine pentoxide itself undergoes a slight decomposition at temperatures below 100° and 150° with evolution of minute traces of iodine (*Proc. Roy. Soc.*, 1909, B. **81**, 515–528).

The fatal accidents due to the inhalation of coal gas, blast-furnace gases, lime-kiln gases, products of combustion of charcoal, or the combustion products that have passed through red-hot iron stoves, or from gas produced in coal-dust explosions in mines, are due to various degrees of saturation of the hæmoglobin of the blood with carbon monoxide.

Experiments by J. S. Haldane (*J. Physiol.*, 1895, **18**, 430) on himself show that the symptoms caused by carbonic oxide depend on the extent to which the hæmoglobin has been saturated. During rest the symptoms do not become sensible till the corpuscles are about one-third saturated. With half saturation, the symptoms (headaches, respiratory distress, etc.), become urgent. About one-half of the carbonic oxide contained in the respired air is absorbed.

The time required for the production of sensible symptoms varies in different animals with the respiratory exchange per unit of body-weight, and is about twenty times as long in a man as in a mouse. Hence a mouse can be used as an indicator of the respirability of a vitiated atmosphere before men venture into it.

With a given small percentage of carbonic oxide in air, a certain percentage saturation of the blood is reached in about 150 minutes, and is not afterward exceeded, however long the breathing of air vitiated to the same extent is continued. Distinct symptoms of poisoning, appreciable during rest, are produced when the proportion of carbonic oxide reaches 0.05%, and with about 0.2% urgent symptoms are produced. The disappearance of the carbonic oxide from blood when fresh air is again breathed is always much slower than its absorption, and is chiefly due to the dissociation of carboxyhæmoglobin by the mass-influence of the oxygen in the pulmonary alveoli.

Nitric Oxide Hæmoglobin, Hb-NO.—This compound was first described by L. Hermann in 1865. Nitric oxide possesses an even greater affinity for hæmoglobin than carbon monoxide, and will displace this from carboxyhæmoglobin. Owing to the great affinity of nitric oxide for oxygen, this compound is best prepared by passing nitric oxide through a solution of carboxyhæmoglobin. In order to carry out the experiment, the solution is placed in a flask with some arrangement for permitting the whole of the air above the solution to be driven out and replaced by a neutral gas before allowing access to the nitric oxide. After the latter has exerted its action, the neutral gas must again be passed through to remove all traces of nitric oxide.

It may be prepared from oxyhæmoglobin in a similar way, but in this case sufficient alkali must be added to the solution to neutralise the nitric acid produced by the action of nitric oxide in the oxygen of the hæmoglobin.

Recently the combination of nitric oxide with haemoglobin has been re-investigated by Anson and Mirsky (*T. of Physiol.*, 1925, **60**, 100). They found that in the reaction between NO and oxyhaemoglobin or carboxyhaemoglobin the first step is the formation of methaemoglobin. A solution of oxy- or carboxyhaemoglobin is injected by means of a syringe into a tonometer containing nitric oxide diluted with hydrogen. The events are followed spectroscopically. If the solution is kept alkaline, methaemoglobin first appears, and this then reacts with nitric oxide to form the ordinary nitric oxide haemoglobin, but which should be called nitric oxide-methaemoglobin, because it is with methaemoglobin that the nitric oxide combines. If, however, the experiment is repeated in slightly acid medium (*p*H 8) acid methaemoglobin first appears, and

nitric oxide reacts with this to form a new compound whose absorptions, spectrum and colour are different from those of alkaline nitric oxide-methaemoglobin. In a few minutes, however, the new form goes over into the alkaline form, but if the solution is now made still more acid, this transformation is reversed and the new form re-appears.

The nitric oxide methaemoglobin proved to be an indicator with a turning point at about *p*H 5.6. In other words, nitric oxide methaemoglobin is of the order of a thousand times as strong an acid as is methaemoglobin, which explains why at certain hydrogen ion concentrations the acid form changes over with the alkaline.

The nitric acid of the alkaline form cannot be removed in a vacuum, but it can easily be pumped off from the acid form.

Blood saturated with nitric oxide possesses almost as florid a colour as CO blood, but does not possess the slight bluish shade of the latter. It is not dichroic. The solutions are very stable, and keep even longer than those of carboxyhæmoglobin.

It can be crystallised; the crystals are identical in form and aspect with those of oxy- and carboxyhæmoglobin. (*Cf.* Hartridge, *Analyst*, 1931, **56**, 571.)

Spectrum.—The bands in the visible spectrum occupy the same position as the two bands of oxyhæmoglobin, and in the photographic spectrum the band in the extreme violet is absolutely coincident with that of carboxyhæmoglobin. The new acid form is browner than the alkaline form and its two-banded spectrum is more distinct. The α band is at 568.7.

Nitric oxide hæmoglobin, so called, is recognised by the following tests. On boiling, a red coagulum is produced which remains unchanged in the air. While oxyhæmoglobin is converted into methæmoglobin by the action of potassium ferricyanide, followed by acetic acid, in the case of the nitric oxide compound, this change proceeds very slowly and the colour remains brownish-red, and if examined with a spectroscope the band of methæmoglobin is seen in the red, together with both the bands of nitric oxide hæmoglobin.

Nitrites are very poisonous when taken by the mouth, and the blood contains a mixture of methæmoglobin, nitric oxide- and oxyhæmoglobin (Haldane, *J. Physiol.*, 1898, **22**, 298). Haldane also found that the red colour of meat pickled by potassium nitrate is due to nitric oxide hæmoglobin (*J. Hygiene*, 1901, **1**, 115).

Cyanhæmoglobin.—According to Preyer, when an aqueous solution of oxyhæmoglobin is treated with potassium cyanide or prussic acid and allowed to stand at room temperature, or is warmed to about 40°, the solution assumes an orange-yellow shade. This can be instantly produced, according to Kobert, if a solution of methæmoglobin is treated with 0.5% hydrogen cyanide. Von Zeynek (*Z.*

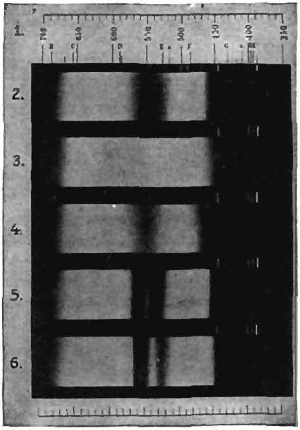


FIG. 11.

1. Scale of wave-lengths.
2. Dilute rabbits' blood, 1:80, treated with ferricyanide of potassium and hydrocyanic acid added.
3. Dilution, 1:80. Converted into hæmatin with sodium hydroxide.
4. Solution 3 plus hydrogen cyanide (cyanhæmatin).
5. Solution 4 plus ammonium sulphide (cyanhæmochromogen).
6. Solution 3 plus ammonium sulphide (hæmochromogen).

physiol. Chem., 1901, **33**, 426–480) found that crystallised methæmoglobin dissolves in 0.5% solution of hydrogen cyanide, yielding a reddish liquid having a characteristic broad absorption band in the green. When diluted and mixed with alcohol at −10° microscopic crystals are obtained. As a rule, these are long prisms, terminated by pyramids which occasionally are rhomboidal in shape. The same compound is also obtained by adding potassium cyanide to

alkaline methæmoglobin solution. The compound is stable, and may be heated to 40° in a stream of indifferent gas, or its solution may be boiled at 40° in vacuo without loss of prussic acid. The compound contains 0.158% of CN, a result indicating the presence of one CN group in the molecule. Boeck's photo-methæmoglobin, obtained by exposing weak solutions of methæmoglobin made by the action of ferricyanide of potassium on oxyhæmoglobin to sunlight, has been shown by Haldane (*J. Physiol.*, 1900, **25**, 230) and von Zeynek to be cyanhæmoglobin.

In Fig. 11 is shown the spectra of hæmatin in alkaline solution, of cyanhæmoglobin, of cyanhæmatin and cyanhæmochromogen (Rost, Franz and Heise).

Reduced hemoglobin does not combine with hydrogen cyanide.

Spectrum.—This resembles hæmoglobin (reduced). Compare Figs. 39, 2, with Fig. 36. Ziemke and Muller give $\lambda = 579 - 520\mu$ for the extent of the band of cyanhæmoglobin and $\lambda = 596\mu\mu$, $\lambda = 543\mu\mu$ for hæmoglobin (*Arch. Physiol.*, 1901, Suppl. Bd., 177).

By the addition of Stokes' reagent or ammonium sulphide, the spectrum undergoes a change. Two bands, all but indistinguishable from those of hæmochromogen, are seen. The bands of cyanhæmochromogen, according to Ziemke and Muller, are of equal density and extend more toward the red part of the spectrum.

In cyanhæmochromogen the bands extend from $\lambda = 577\mu\mu$ to $\lambda = 562\mu\mu$, and from $\lambda = 548\mu\mu$ to $\lambda = 592\mu\mu$.

Sulphhæmoglobin, or Sulphmethæmoglobin.—This compound was believed by Hoppe-Seyler to be the cause of the greenish colour of the skin of corpses. This is well seen on the abdomen. It is produced by passing hydrogen sulphide through blood and in putrefaction by the formation of this gas by bacteria. The solution is green in thin layers, red in thick layers, and somewhat turbid. Such a solution shows a well-marked absorption band $\lambda = 610\mu\mu - 622\mu\mu$. Gamgee considers there is not the slightest ground for believing that the spectrum described is due to a definite body, but is caused by the decomposition products of oxyhæmoglobin. (*Text-book of Physiology*, Ed. by Schäfer, **1**, 251, 1898.)

Clarke and Hurtley (*J. Physiol.*, 1907, **36**, 62) regard sulphhæmoglobin as a definite compound and not the result of any decomposition products of oxyhæmoglobin. They draw attention to a characteristic test for this substance. When acid-free carbon mon-

oxide is passed into a solution of sulphhæmoglobin, all the band are shifted toward the violet. The bands of oxyhæmoglobi become those of carboxyhæmoglobin, while the band in the re moves from $\lambda = 610\mu\mu$ to $\lambda = 625\mu\mu$ and $\lambda = 605\mu\mu$ to $\lambda = 620\mu\mu$ This is due to the production of a new compound, COSHb.

Van den Bergh and Wieringen state that hydrogen sulphid combines with haemoglobin or reduced haemoglobin only in th presence of oxygen. It has no effect on reduced haemoglobin i air be completely excluded.

Hydrogen selenide behaves in a similar way to hydrogen sulphide in this case the band apparent is $\lambda = 613\mu\mu - \lambda 628\mu\mu$ (selenhæmo globin).

Methæmoglobin, Met.-Hb.—It was observed as long ago as 1864 by Hoppe-Seyler, that solutions of oxyhæmoglobin on exposure t the air assume a brown colour, become acid, and give a spectrum i which, in addition to the two bands of oxyhæmoglobin, a band i seen in the red in much the same position as the band of acid hæma tin. He called the indefinite substance in such solution methæmo globin, and considered it to be a product of the partial reduction o oxyhæmoglobin and derived from it by the removal of a portion o the dissociable oxygen. It has since been discovered that a larg number of substances—organic and inorganic, oxidising agents an reducing agents—act upon solutions of oxyhæmoglobin, convertin them into methæmoglobin. Such substances are: potassium ferri cyanide, hydroxylamine, nitrites, chlorates, iodine, formaldehyde nitrobenzene, pyrogallol, pyrocatechol, acetanilide, etc., and also th β-rays of radium. Even violent agitation with mercury will brin about some degree of change. In order to study the spectroscopi character of methæmoglobin, a solution of diluted blood is treate with a few drops of a strong solution of potassium ferricyanide o shaken with a few crystals, when the change in colour and spectrun is seen to occur almost instantly. In order to prepare the crystallin colouring matter, 2–3 c.c. of a saturated solution of potassium ferri cyanide or of a nitrite are added to a litre of saturated aqueous solu tion of crystals of oxyhæmoglobin, and after the conversion has take place, alcohol is added to the extent of 25%. The liquid is then lef to stand in a chamber below 0°, when crystals gradually separate The crystals are more difficultly soluble in water, and form mor readily than is the case for oxyhæmoglobin.

When oxyhæmoglobin is changed into methæmoglobin, the oxygen is not removed, but passes into such a condition that it can no longer be removed by boiling *in vacuo*, or by the action of carbon monoxide, though it can be replaced by nitric oxide to produce nitric oxide hæmoglobin.

The substances which convert oxyhæmoglobin into methæmoglobin do not act catalytically (Babel, *Arch. Sc. phys. nat.*, 1896; series 4, **22**, 216–239), because, if care be taken to avoid secondary action, it is not possible to recover the whole of such substances at the end of the reaction, and further, there exists for each substance a limiting quantity, below which it is incapable of effecting any change in the oxyhæmoglobin. In the change of oxy- to methæmoglobin by the action of potassium ferricyanide, Haldane (*J. Physiol.*, 1898, **22**, 298) has shown that a volume of oxygen equal to the volume of the oxygen combined in oxyhæmoglobin is evolved. The action may be represented by the equation:

$$2(K_3Fe'''(CN)_6) + \begin{matrix}H{-}OH\\ H{-}OH\end{matrix} = 2(K_3HFe''(CN)_6) + \begin{matrix}OH\\ OH\end{matrix}$$

$$Hb\left\langle\begin{matrix}O\\ |\\ O\end{matrix}\right. + \begin{matrix}OH\\ OH\end{matrix} = Hb\left\langle\begin{matrix}OH\\ OH\end{matrix}\right. + O_2$$

The formula $Hb\left\langle\begin{matrix}OH\\ OH\end{matrix}\right.$ for methæmoglobin is open to the objection that the acid characters of methæmoglobin are not sufficiently marked, and Haldane prefers the formula $Hb\left\langle\begin{matrix}O\\ O\end{matrix}\right.$.

Methæmoglobin will also combine with nitric oxide. Hüfner and Reinbold (*Archiv. f. Anat. u. Phys.*, 1904, Supp., Bd., 391) have determined for this compound that 1 grm. of methæmoglobin associates 2.66 c.c. of oxygen at 0° and 760 mm., exactly double the quantity of oxygen or carbonic oxide associated with 1 grm. of hæmoglobin or hæmochromogen.

Reducing agents, *e.g.*, Stokes' reagent, convert methæmoglobin into hæmoglobin. According to Gamgee, this change is preceded by the momentary formation of oxyhæmoglobin.

Conant, Conant and Fieser, Conant and Scott, in a series of papers between 1923 and 1928, have shown that it is characteristic of methaemoglobin that by proper reducing agents it is transformed into reduced haemoglobin, with restoration of its ability to combine with oxygen and carbon monoxide. It was shown that the change of haemoglobin to methaemoglobin involves one equivalent of oxidising agent per iron atom of the hæmoglobin molecule, and that the oxidation of haemoglobin to methaemoglobin and the reduction of the latter to haemoglobin appears to be a strictly reversible change, and may be formulated according to the principles of electro-chemistry.

Hill and Holden (*Biochem. J.*, 1926, 1326) 20, have prepared methaemoglobin by the direct union of haematin and globin.

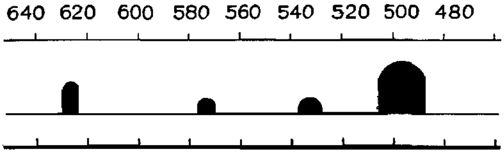


FIG. 12.—Neutral methæmoglobin.

Solutions of hæmoglobin and oxyhæmoglobin are not precipitated by either neutral or basic lead acetate, but these reagents, if added cautiously so as to avoid excess, precipitate methæmoglobin, hæmatin and hæmatoporphyrin, and may be used for the separation and detection of traces of oxyhæmoglobin when mixed with and concealed by any of the above-mentioned bodies.

Spectrum.—The characters of the spectrum of methæmoglobin have been a matter of considerable controversy, since different methods have been employed in the production of this body. In blood that has stood some weeks exposed to the air, or when the bloody fluids of the cavities of the thorax or abdomen of corpses are examined, the spectrum generally shows a feeble absorption band in the red, two wider bands occupying a position similar to those of oxyhæmoglobin, and a fourth band in the green-blue region. A similar spectrum is seen when methæmoglobin is made artificially by the reagents mentioned above and is given by pure solutions of methæmoglobin

crystals. It is certain that if the solutions of methæmoglobin are neutral the first and fourth bands belong to methæmoglobin; but it is a disputed matter, as Lewin, Formanek (*Z. analyt. Chem.*, 1901) and others contend, whether the two middle bands represent the spectrum of unchanged oxyhæmoglobin, or are features of methæmoglobin itself. The weight of evidence appears to favour the view that only the two extreme bands, the first and the fourth, are characteristic of methæmoglobin, and that when animals are poisoned with nitrites or hydroxylamine, just as in carbon monoxide poisoning, a part only of the hemoglobin is converted into methæmoglobin. (See Appendix.)

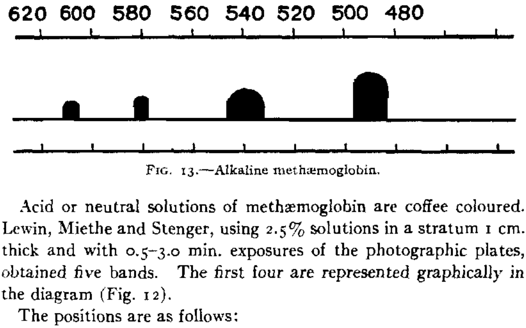


Fig. 13.—Alkaline methæmoglobin.

Acid or neutral solutions of methæmoglobin are coffee coloured. Lewin, Miethe and Stenger, using 2.5% solutions in a stratum 1 cm. thick and with 0.5–3.0 min. exposures of the photographic plates, obtained five bands. The first four are represented graphically in the diagram (Fig. 12).

The positions are as follows:

Met-Hb	$Hb\text{-}O_2$
$\lambda = 626\mu\mu$	
$\lambda = 575\mu\mu$	$\lambda = 577\mu\mu$
$\lambda = 533\mu\mu$	$\lambda = 537\mu\mu$
$\lambda = 499\mu\mu$	
$\lambda = 410\mu\mu$ (violet band)	

The violet band was taken using 1% solution in a stratum 1 cm. thick. The second and third bands are to be regarded as the bands of oxyhæmoglobin, although they are slightly shifted toward the violet end of the spectrum.

An alkaline solution of methæmoglobin is chestnut brown, and the absorption bands characteristic of it are somewhat different in form and position. This is graphically represented in Fig. 13.

The positions are different from those of neutral methæmoglobin. The five bands are:

Alkaline met-Hb	HbO_2
$\lambda = 608\mu\mu$	
$\lambda = 578\mu\mu$	$\lambda = 570\mu\mu$
$\lambda = 540\mu\mu$	$\lambda = 540\mu\mu$
$\lambda = 493\mu\mu$	
$\lambda = 415\mu\mu$	

The second and third bands correspond with those of oxyhæmoglobin.

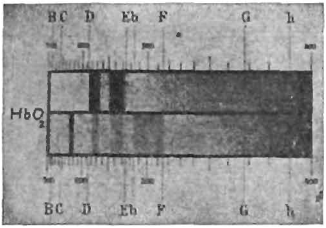


FIG. 14.

In Fig. 14, taken from Bürker, we give the comparative spectra of HbO_2 and neutral methæmoglobin, and in Fig. 15 that of HbO_2 and alkaline methæmoglobin.

The spectrum of methæmoglobin is not always easy to recognise, and the observation of Kobert, that the addition of hydrogen cyanide or cyanogen gas to methæmoglobin forms cyanmethæmoglobin, renders this reaction of considerable diagnostic importance. The brown-colour of the solutions or of the organs of corpses change to red.

The spectra of methæmoglobin and acid hæmatin are very similar, each showing a band in the red. The acid hæmatin spectrum remains

unchanged on the addition of Stokes' reagent, that of methæmoglobin is replaced by the spectrum of reduced hæmoglobin.

In recent years methods have been developed by Nicloux and Fontis (*Bull. Soc. Chim. Biol.*, 1924, **6**, 728), Conant and Fieser (*J. Biol. Chem.*, 1924–25, **82**, 223), and by Conant, Scott and Douglass (*J. Biol. Chem.*, 1928, **76**, 223), for the estimation of methaemoglobin, based on the reduction of methaemoglobin to haemoglobin, and the estimation of the latter gasometrically. For details, however, the original papers must be consulted.

Fluormethæmoglobin.—The addition of alkaline fluorides or fluor-silicates to solutions of methæmoglobin causes the band in the red to shift toward the yellow ($\lambda = 612 \mu\mu$). This so-called "band of Menzies," even if the band in the methæmoglobin is not very

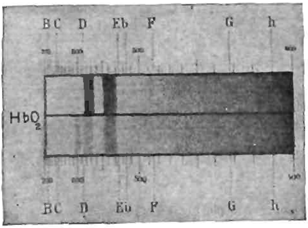


FIG. 15.

marked, is, in great dilution, readily seen, and can be used for the identification of methæmoglobin. Fluormethæmoglobin can also be crystallised, but the crystals are unstable (*J. Physiol.*, 1895, **17**, 402–422).

The spectrophotometer constants are given on page 29. See Table 3.

The Products of the Hydrolysis of Hæmoglobin.—Oxyhæmoglobin has hitherto been regarded as a salt or perhaps ester-like substance, and, when treated in aqueous solution with dilute acids or alkalies, or when the solution is simply warmed to 64–69°, breaks up into globin—a histone of basic character—and hæmatin, a colouring matter of acid character. Small quantities of simple substances, such as formic acid, butyric acid, etc., are also formed.

Schulz (*Z. physiol. Chem.*, 1898, **24**, 449) from pure oxyhæmoglobin obtained 86.5% of globin, 4.2% of hæmatin and 9.3% of indeterminate matter. Lawrow (*Z. physiol. Chem.*, 1898, **26**, 343), however, obtained 94.09% proteins, 4.47% hæmatin and only 1.44% of other constituents.

The most important derivatives related to hæmatin are hæmin, hæmochromogen and hæmatoporphyrin.

Within recent years, chiefly as a result of the work of Anson and Mirsky (*J. physiol.*, 1925, **60**, 161; **60**, 221; **60**, 50), the views of physiologists on the relation of haemoglobin to haematin and haemochromogen have undergone considerable changes, and even now may be said to be in a state of transition. According to Anson and Mirsky, who worked chiefly from a spectroscopic standpoint, haemoglobin can exist as such near the neutral point only. Diluted acid or alkali changes it into haemochromogen. This change is not, as hitherto interpreted, a separation of haem from globin, and haemochromogen is not a reduced form of haem. This separation does not occur, and haemochromogen, and hence its oxide haematin, is a conjugated protein—a globin compound. Evidence that haemochromogen is not simply reduced haem is that it can be synthesised from reduced haem by adding a protein to it. When a few crystals of haemin are dissolved in dilute sodium hydroxide solution and a little sodium hydrosulphite added as a reducer, reduced haem is formed—a substance whose absorption, spectrum, colour and solubility are quite different from those of haemochromogen. If now globin is added, the substance formed is indistinguishable from the haemochromogen prepared by the action of sodium hydroxide on reduced haemoglobin; other proteins, amino-acids, amines, ammonia, hydrzine hydrate, pyridine, nicotine, pyrrol and other bases can be used instead of globin. All these artificial haemochromogens have similar, but not identical, properties; for example, taking globin-haemochromogen as zero, nicotine-haemochromogen is 3.5 Å and glycocoll-haemochromogen 21.5 Å to the blue, while, taking CO-globin-haemochromogen as zero, CO-nicotine haemochromogen is 16 Å to the red and CO-glycocoll-haemochromogen 7 Å to the red. Globin is only one of many substances which will perform the same function, thus forming a whole group of haemochromogens

Haemin and sodium hydroxide ↗ Haematin
↘ Haem and ammonia ⇄ NH_3-haemochromogen.
Haem and glycocoll ⇄ Gl-haemochromogen.
Haem and hydrazine ⇄ H-haemochromogen.
Haem and pyridine ⇄ P-haemochromogen.
Haem and albumin ⇄ A-haemochromogen.
Haem and globin ⇄ G-haemochromogen.

All these haemochromogens seem to be kept *in statu quo* as the result of balanced reaction, and, as a fact, for the production of most of them a great preponderance of the components is necessary.

The globin-haemochromogen stands alone in that the action is nearly complete. This compound is also one of the few haemochromogens which have a high degree of solubility, and it alone can form a haemoglobin.

Haemin + NaOH ↗ Haematin
↘ Haem + Globin ⇄ Globin-haemochromogen.
⇄ Haemoglobin.

Anson and Mirsky, by regulation of the hydrogen ion concentration, obtained haemoglobin from globin-haemochromogen.

For further information about their views the original papers must be consulted. An excellent account, however, will be found in a lecture on Haemoglobin given before the English Chemical Society by Mr. J. Barcroft, *Trans. Chem. Soc.*, 1926, p. 1146, and in *Physiological Reviews*, 1925, Vol. V, No. 4, p. 596. A review of still more recent work on Haemogobin, the Haem pigments and cellular respiration by M. L. Anson and A. E. Mirsky will be found in *Physiological Reviews* 1930, 10, 506.

Hæmatin, $C_{34}H_{34}O_5N_4Fe$

Hoppe-Seyler prepared hæmatin by the action of strong acetic acid on blood in the presence of ether. From the ethereal solution, after

filtration, the hæmatin separates as an amorphous precipitate. It is, however, difficult to prepare the pure substance from blood, and hæmin is now used as the starting material.

The hæmin is dissolved in the cold in a 10% solution of sodium hydroxide, and the hæmatin formed is precipitated by exact acidification with hydrochloric acid and well washed with hot water.

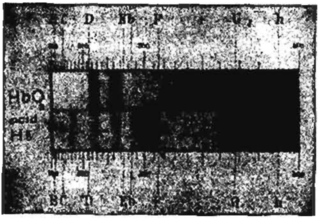


Fig. 16.

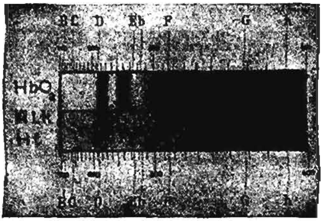


Fig. 17.

It is an amorphous blue-black substance, insoluble in water, alcohol, chloroform or aqueous solutions of acids, but easily soluble in acetic acid, alcoholic solutions of acids, alkalies, and pyridine. It has not hitherto been obtained in the crystalline form. It can be heated to 180° without decomposition, but above this temperature carbonizes with the production of some hydrogen cyanide and pyrrole.

The calcium and barium salts are precipitated when ammoniacal solutions of hæmatin are treated with calcium or barium chloride. The acid solutions are brownish-red in colour, but the alkaline solu-

tions are olive-green and are dichroic. The spectrum in acid solution is different from that in alkaline solution.

Figures 16 and 17 taken from Bürker show the spectrum of an acid alcoholic solution of hæmatin compared with oxyhæmoglobin (Fig. 16) and the comparative spectra of HbO_2 and alkaline hæmatin (Fig. 17).

Acidified blood, or acidified oxyhæmoglobin solutions show in suitable concentration a characteristic absorption band in the red, which in concentrated solution widens out over the whole of the outer part of the red. Using a 0.18% solution of oxyhæmoglobin acidified with hydrochloric acid in a layer of 1 cm., a slit of 0.2 cm. and a variable exposure of 2 to 8 minutes, Lewin, Miethe and Stenger find a maximal absorption at $\lambda = 659\mu\mu$. In the case of alcoholic or acetone solutions of pure hæmatin, rendered acid with hydrochloric acid, a marked displacement of the maximal point was observed. In alcoholic acid solution it was at $\lambda = 632\mu\mu$ and in acetone solution at $\lambda = 630\mu\mu$. Besides this, the spectrum contains three further bands, a faint band near D with a maximum point at $\lambda = 578\mu\mu$, a wider one near E at $\lambda = 540\mu\mu$, and a still wider one near F at $\lambda = 502\mu\mu$.

In the ultra-violet portion of the spectrum, acid blood shows a band $\lambda = 390\mu\mu$. Using an acetone solution of pure hæmatin, the point of maximal absorption is $\lambda = 402\mu\mu$. For alkaline hæmatin, using 0.1 grm. hæmatin dissolved in 15 c.c. alkaline water, a slit of 0.1 mm., a Nernst lamp as a source of light and an isokol plate, the above-mentioned observers find the maximal absorption in the characteristic band at $\lambda = 616\mu\mu$. Besides this there are two further bands, *difficult to photograph and difficult to measure. Their positions are* $\lambda = 568\mu\mu$ and $\lambda = 540\mu\mu$. If acetone solution is used a remarkable displacement of all the bands is noticed. The three corresponding positions of the maximal points are now $\lambda = 580\mu\mu$, $\lambda = 560\mu\mu$ and $\lambda = 524\mu\mu$.

In the ultra-violet part of the spectrum there is a single band, with a maximal point in an aqueous alkaline solution $\lambda = 428\mu\mu$, and in acetone solution $\lambda = 380\mu\mu$.

For a further discussion of the properties of alkali hæmatin and acid hæmatin, the reader is referred to a paper by D. Keilin *Proc. Roy. Soc.* B 1926, **100,** 129. The chemistry of hæmatin is still obscure.

Hæmin, $C_{34}H_{32}O_4N_4FeCl$

This substance is of considerable theoretical and practical importance, and may be regarded as hæmatin in which an (OH) group has been replaced by chlorine. Hæmin may be prepared by any of the following methods:

(1) *Nencki and Sieber's Method* (*Archiv. f. Experim. Path. u. Pharmak.*, 1884, **18**, 401).—Blood corpuscles are washed with physiological saline, and coagulated by alcohol. The moist material is then boiled with amyl alcohol with the addition of hydrochloric acid. The crystals, which separate on cooling, are washed successively with alcohol, chloroform, and water. Yield: 1 grm. from 1 litre of blood.

(2) *Method of Nencki and Zaleski* (*Z. physiol. Chem.*, 1900, **30**, 384). An improvement of a method originally devised by Schalfejeff. 1 litre of glacial acetic acid, saturated at ordinary temperature with sodium chloride, is heated to 90-95°, and 200 c.c. defibrinated blood added with stirring. After 10 minutes the liquid is again heated to 90° and filtered. The crystals, which separate from the filtrate on cooling, are washed with water, then with 65% alcohol, and dried in a vacuum. The dried crystals are then recrystallised from 50 c.c. of the following mixture: 15 parts of 95% alcohol, 4 parts water, and 1 part of an aqueous solution of ammonia (sp. gr. 0.91). The crystals dissolve in this at room temperature in about 30 minutes. The solution is added to 4 to 6 times its volume of glacial acetic acid, saturated with salt, and kept at 110°. On cooling, crystals separate, which are washed with 2% hydrochloric acid and dried *in vacuo*. The yield is 3–4 grm. per litre of blood. The product is called acethæmin.

(3) *Method of K. A. N. Mörner* (Küster, *Z. physiol. Chem.*, 1900, **29**, 187).—1 litre of blood is boiled with 3 litres of water and 10 c.c. of 1% sulphuric acid. The coagulum so produced is stirred with half a litre of 90% alcohol, then filtered and pressed. It is then ground with 1.75 litres of 90% alcohol, to which 35 c.c. of a mixture of equal parts of concentrated sulphuric acid and 90% alcohol are added, and allowed to digest for 1 hour at room temperature. The liquid is strained off and squeezed out of the solid matter, and raised to the boiling-point. To the boiling liquor hydrochloric acid and alcohol are now added in the proportion of 8 c.c. of 25% acid and 12 c.c. of 90% alcohol to every litre of blood, and the whole cooled in

water. After standing two days, the crystals which have been deposited are filtered off, washed with alcohol, then with water and dried. The dried crystals are finally washed with petroleum ether. The yield is 3 grm. per litre of blood. Mörner named the product β-hæmin, as it differed somewhat from other products.

(4) *Method of von Zeynek* (*Z. physiol. Chem.*, 1900, **30**, 126; 1906, **49**, 472).—In this method the protein constituent of hæmoglobin is got rid of by digestion with pepsin and hydrochloric acid. The product is then treated as in Nencki and Zaleski's process.

The hæmins prepared by these various methods were supposed to be different, but Küster (*Z. physiol. Chem.*, 1904, **40**, 391) has shown that the differences were due to impurities, and they consist of one and the same hæmin. Hæmin is most readily purified by warming in chloroform containing quinine (1 grm. hæmin in 50 c.c. chloroform containing 1 grm. of quinine) until dissolved. The solution is then filtered, and on treatment with alcohol containing hydrochloric or acetic acid to neutralise the quinine, hæmin crystallises out. Pyridine may be advantageously used instead of quinine (1 grm. hæmin in 20 c.c. chloroform and 2 c.c. pyridine).

Hæmin is a blue-black crystalline powder with a metallic lustre. It is insoluble in water, alcohol or ether, slightly in dilute mineral acids, alcohol containing hydrochloric or acetic acid, and fairly soluble in solutions of alkalies and alkaline carbonates.

The change of hæmin into hæmatin takes place according to the equation

$$C_{34}H_{33}N_4FeClO_4 + KOH = C_{34}H_{34}N_4FeO_5 + KCl.$$

The reverse change of hæmatin into hæmin can be brought about quantitatively by adding a solution of hæmatin in chloroform containing quinine to a hot glacial acetic acid solution of sodium chloride, and stirring well. Crystals of hæmin separate on cooling. After repeating the above operation, steel-blue glistening crystals of pure hæmin are obtained (O. Piloty, *Annalen*, 1910, **377**, 314).

Hæmin contains two carboxyl groups and a number of esters have been prepared (Nencki and Zaleski, *Z. physiol. Chem.*, 1900, **30**, 384; Küster, *Ber.*, 1910, **43**, 2960). The dialkyl ethers, unlike hæmin, are insoluble in alkalies, and their nitrogen can be split off as ammonia, so that the alkyl groups are not in combination with the nitrogen. Hæmin, on treatment with aniline, loses the elements of

hydrogen chloride and yields, after extraction with dilute acetic acid and ether, an amorphous product—*dehydrochlorhæmin* ($C_{34}H_{32}O_4N_4Fe$), which, if fresh, can be converted again into hæmin by the action of hydrochloric acid, but, on keeping, it undergoes some change which prevents this.

Dehydrochlorhæmin, when dissolved in alkali and reprecipitated by acid, is transformed into an isomeride dehydrohæmatin, since it is no longer capable of being converted into hæmin.

Hæmin forms salts with three molecules of alkali hydroxide and dehydrochlorhæmin with two molecules. These salts can be dialysed in 1% solution without any pigment coming through. Hæmin only takes up two molecules of sodium carbonate, and sodium hydrogen carbonate appears in the outer water on dialysis. On precipitating these alkali salts by means of solutions of other metallic salts, the precipitates show very varying quantities of metal. Both hæmin and hæmatin are insoluble in acid carbonate and di-acid phosphates, but with normal carbonate, acid carbonates are formed. The di-sodium salt of hæmatin gives up a molecule of sodium hydroxide on prolonged dialysis. Solutions of the alkali salts of hæmatin polymerise on keeping. The two acid hydroxyl groups in hæmin are also present in hæmatin, though so far no hæmatin esters have been prepared. The third hydroxyl in hæmatin which is readily replaced by chlorine, is removed when iron is withdrawn from the hæmatin molecule, and is, therefore, presumably attached to the iron atom.

The ready formation of hæmin from hæmoglobin is taken advantage of in Teichmann's well-known test for blood stains. (See p. 105.) The spectrum of hæmin in alkaline or acid solution corresponds with that of hæmatin in like solvents.

Hæmochromogen, $C_{64}H_{64}N_8Fe_2O_7$

This substance—the reduced alkaline hæmatin of Stokes—is formed when blood or a solution of methæmoglobin is boiled with a dilute solution of potassium hydroxide, or treated in the cold with 32% potassium hydroxide or concentrated sodium hydroxide. These operations must be carried out in an atmosphere free from oxygen. Beautiful ruby red crystals are obtained as a microscopic preparation when a drop of blood is mixed with pyridine on a slide and then ammonium sulphide added. The crystals are most easily obtained by mixing a drop of fresh blood with an equal amount of pure pyri-

dine, then adding but not mixing a drop of 50% hydrazine sulphate, covering and quickly raising just to the boiling-point. The preparation must not be boiled. For other methods see Nippe (*Deutsche med. Wochensch.*, Nov., 1912, No. 47); also W. J. Dilling, *Atlas der Kristallformen und d. Absorptionsbänder der Hæmochromogen*, Stuttgart, 1910. (Fig. 18.) Pure hæmatin is not reducible, but hæmochromogen is formed when it is reduced by Stokes' reagent (see this) or hydrazine hydrate in presence of a trace of albumin. It is also formed when hæmatin is reduced by means of potassium hydro-

FIG. 18.

gen sulphide. It is not possible to eliminate the oxygen of hæmatin in the blood-pump to produce hæmochromogen. If hæmatin is suspended in alcoholic ammonia and reduced with hydrazine in an atmosphere of hydrogen, and precipitated by ether (still in a hydrogen atmosphere) a pure ammonium compound of hæmochromogen is obtained. v. Zeynek (*Z. physiol. Chem.*, 1898, **25**, 492) considers that in the reduction of the hæmatin one atom of oxygen is eliminated from two molecules, and the two hæmatin residues combine together with two NH_4 groups, forming $C_{64}H_{72}$-$O_7N_{10}Fe_2$. Hæmochromogen itself would then be $C_{64}H_{72}O_7N_8Fe_2$. According to Küster, the iron of the hæmochromogen is in the ferrous state (*Z. physiol. Chem.*, 1910, **66**, 165).

The spectrum of hæmochromogen is exceedingly characteristic. It consists of two bands, one a dark, sharply defined band between the

position of the bands of oxyhæmoglobin, and a much feebler, somewhat wider band about E. Lewin, Miethe and Stenger, using hæmochromogen obtained from oxyhæmoglobin through hæmatin, give the following situations of the maximal absorption in the bands: $\lambda = 556\mu\mu$ and $\lambda = 530\mu\mu$, and, using hæmochromogen from pure hæmatin $\lambda = 558\mu\mu$ and $\lambda = 526\mu\mu$. The band in the ultra-violet is also characteristic, and persists in dilutions in which the other bands cannot be distinguished. Gamgee gives the position between G and

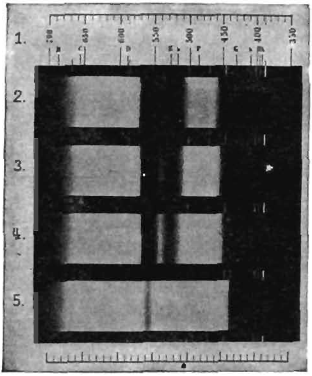


FIG. 19.—From Franz, Rost and Heise.
1. Scale of wave lengths. 3. Dilution, 1 : 80.
2. Dilution, 1 : 60. 4. Dilution, 1 : 100.
5. Dilution, 1 : 300.

h = $420\mu\mu$. Lewin, Miethe and Stenger give $\lambda = 411\mu\mu$. The spectrum of hæmochromogen is of high diagnostic value, for if the solution under examination consists of a mixture of oxyhæmoglobin, methæmoglobin and hæmatin, and the spectrum is consequently of a mixed character, since all these substances pass under suitable reduction into hæmochromogen, which is the basic substance of this colour group, the well-defined absorption spectrum of this substance can be produced. Further, as the dark band is very persistent, it often appears in the reduction of solutions too dilute to show the bands of hæmoglobin or hæmatin.

In Fig. 19 we give the spectrum of solution of rabbit's blood converted into hæmatin by an alkali and reduced to hæmochromogen by ammonium sulphide.

Hæmochromogen forms compounds with carbon monoxide, nitric oxide and the cyanogen group.

Carboxyhæmochromogen.—If an ammoniacal solution of hæmin is reduced with hydrazine hydrate and saturated with carbon monoxide in an atmosphere of this gas, and the solution so obtained mixed with an equal volume of a concentrated sodium chloride solution also saturated with carbon monoxide, a compound of hæmochromogen and carbon monoxide is precipitated. The precipitate is washed with half saturated sodium chloride solution, then with alcohol, finally with ether, and then dried. All the operations must be conducted in an atmosphere of carbon monoxide free from air. The dry powder of CO-hæmochromogen is stable in dry air, but in moist air changes to hæmatin (Pregl, *Z. physiol. Chem.*, 1905, **44**, 173).

Linossier, and also Gamgee, obtained nitric oxide hæmochromogen by the action of nitric oxide on hæmochromogen, but this substance has not been purified. Haldane also found it in meat salted with nitrates.

Cyanhæmochromogen is obtained by adding 0.5% aqueous prussic acid to alkaline hæmatin and reducing the cyanhæmatin with ammonium sulphide. It has never been purified.

Hæmatoporphyrin, $C_{34}H_{38}O_6N_4$ (P. Eppinger) *Philos. Dissertations*, 1907, München

When blood or hæmoglobin, or any of its iron-containing derivatives—moist or dry—is mixed with concentrated sulphuric acid and warmed on the water-bath, iron is split off from the hæmatin molecule with the production of a beautiful red-green solution of acid hæmatoporphyrin. If this solution is poured into water, hæmatoporphyrin is precipitated in the form of brown flocks. Laidlaw has shown that the iron is much more readily split off from reduced than from oxyhæmoglobin (*J. Physiol.*, 1904, **31**, 464).

Nencki and Zaleski (*Z. physiol. Chem.*, 1900, **30**, 423; and *Annalen*, 1910, **377**, 314) recommend the following method of preparation from hæmin:

5 grm. of hæmin (it is best to work with small quantities) are added gradually with vigorous shaking, to 75 c.c. of glacial acetic acid pre-

viously saturated at 10° with hydrogen bromide. The liquid is allowed to stand at room temperature for 3 or 4 days with occasional shaking. When all the hæmin has dissolved and the solution acquired the beautiful red tint of hæmatoporphyrin, the contents of the flask are poured into distilled water, and after standing several hours are filtered. The filtered solution is now treated with sodium hydroxide solution, until all the hydrobromic acid is neutralised, when the colouring matter, which is quite insoluble in dilute acetic acid, is precipitated. The precipitate is allowed to settle and thoroughly washed, at first by decantation, until the filtrate no longer reacts with silver nitrate. The washed but still moist hæmatoporphyrin is then digested on a water-bath with a pure dilute solution of sodium hydroxide for 15 minutes, and the coloured solution filtered from the precipitated oxide of iron. The colouring matter is then precipitated from the filtrate by means of acetic acid, again filtered off and well washed. The precipitate is now made into a paste with a little water in a dish, and ground up with small portions of hydrochloric acid until the colouring matter goes into solution. The solution is then filtered from any resinous matter and more hydrochloric acid added. Should any more resin be precipitated, it is again filtered and then evaporated *in vacuo* over sulphuric acid until crystals of hæmatoporphyrin hydrochloride separate. The crystals are separated from the mother-liquors on the pump and well washed with 10% hydrochloric acid. After again recrystallising, the hæmatoporphyrin hydrochloride is obtained in the pure state.

Pure hæmatin, on standing with a glacial acetic solution of hydrogen bromide at room temperature, also gives hæmatoporphyrin.

Of the many variations of the original method, that of Willstätter and Fischer (*Z. Physiol. Chem.*, 1913, **87**, 461) is perhaps the best because in this method haematoporphyrin was *first obtained in crystalline condition.* 36 grm. haemin crystals are added to 900 grm. of glacial acetic acid, into which hydrogen bromide had been passed until the specific gravity at 0° was 1.41, and shaken for ¼ hour. The mixture is allowed to stand for 7 hours until a clear solution is obtained. It is then poured into 5 litres of water, filtered from any particles through a thin layer of talc, and allowed to stand a further three hours. The haematoporphyrin is then precipitated by the addition of concentrated sodium acetate solution. This is

filtered and washed with water until the wash waters give no reaction for hydrobromic acid with silver nitrate. For purification the still moist precipitate is dissolved in quite dilute caustic soda solution, filtered from iron hydroxide and the colouring matter again precipitated from the solution with acetic acid. The precipitate is then filtered by the aid of a pump. To obtain crystalline haematoporphyrin the still moist mud is dissolved in alcohol, in the proportion of one litre to every 10 grams of haemin used, and the solution added to 25 litres of ether. The alcohol is then quickly washed out by water and the ethereal solution dried with anhydrous sulphate of soda and evaporated to 1 litre. Even when the solution is warm crystallisation begins and is completed at warm temperatures. The yield is about half the haemin used.

The crystals of free haematoporphyrin consist of long leaves with rounded ends, of violet colour, but brown in transmitted light. They are difficultly soluble in ether, methyl and ethyl alcohols, but dissolve more easily in acetone and in glacial acetic acid.

The *hydrochloride*, $C_{34}H_{38}O_6N_4$, 2HCl, of hæmatoporphyrin forms star-shaped aggregates of needles. It is readily soluble in dilute hydrochloric acid, but the solubility decreases with the strength of acid. On treating a solution with sodium acetate the hæmatoporphyrin is thrown out of solution in brown amorphous flocks which, after drying in the cold, have the composition $C_{34}H_{38}O_6N_4$. Hæmatoporphyrin in amorphous state is easily soluble in a solution of alkali hydroxides and alkali carbonates, dilute mineral acids, and alcohol, but with difficulty soluble in ether, amyl alcohol and chloroform. It is insoluble in water and dilute acetic acid, and is precipitated in acid solution by means of sodium chloride, magnesium chloride, ammonium sulphate and lead acetate.

Solutions of hæmatoporphyrin in acidified alcohol have a beautiful purple colour, and assume a bluish-violet tint when the solution is made strongly acid. Alkaline solutions are a fine red, but with an excess of alkali become more violet in tint. Solutions of hæmatoporphyrin also, even if extraordinarily dilute, exhibit a magnificient red fluorescence.

Hæmatoporphyrin behaves as an amino-acid with two secondary alcoholic hydroxyl groups. Dimethyl- and diethyl-ethers and a monoacetyl derivative have been prepared (Nencki and Zaleski, *Z. physiol. Chem.*, 1900, **30**, 429). For a more recent account of the

ethers and esters, see papers by Willstätter and Fischer, Küster and Geering, and Küster and Bauer (*Z. physiol. Chem.*, 1922, **109**, 125: 1915, **99**, 172). The presence of ethylene linkages is proved by the readiness with which it is reduced by sodium amalgam, one molecule of the compound taking up 6 to 8 atoms of hydrogen. The solution of the leuco-base thus obtained reduces Fehling's solution, and ammoniacal silver nitrate, and on addition of acid yields a white precipitate of the base, which turns brown on exposure to air. The leuco-base cannot be prepared in the pure state, but on oxidising the solution by air and acidifying the product, a solution is obtained which appears to be dioxyhæmatoporphyrin (Piloty and Stein, *Annalen*, 1910, **377**, 314).

Spectrum.—The spectra of acid and alkaline hæmatoporphyrin differ from one another in the position of their absorption bands, and, further, both products show differences according as the solution examined is prepared from animal and human blood or from pure previously prepared hæmatoporphyrin. According to Lewin, a hæmatoporphyrin solution prepared from blood by means of concentrated sulphuric acid shows 3 absorption bands, one in the orange, a second feeble one, and a third in the green. Lewin, Miethe and Stenger give the positions of maximum absorption as $\lambda = 598\mu\mu$, $\lambda = 575\mu\mu$ and $\lambda = 533\mu\mu$. In the violet there is a band $\lambda = 404\mu\mu$.

If pure hæmatoporphyrin hydrochloride is used in aqueous hydrochloric acid solution, the 3 bands above mentioned are seen, but if a solution of the dry hydrochloride in alcohol is used there are 5 bands, but two vanish again on adding a mineral acid. A specimen obtained by Miethe, Lewin and Stenger from Frau Sieber showed in aqueous hydrochloric acid solution 6 bands, of which the first four were easily recognisable, but the other two in the green required special care for recognition. The positions of the maximal absorption of the 6 bands are $\lambda = 593\mu\mu$, $\lambda = 571\mu\mu$, $\lambda = 550\mu\mu$, $\lambda = 540\mu\mu$, $\lambda = 520\mu\mu$, and $\lambda = 510\mu\mu$. The two bands in the violet and ultra-violet are at $\lambda = 403\mu\mu$ and $\lambda = 380\mu\mu$. According to Willstätter in alcoholic solutions the spectrum is dependent on the degree of dissociation of the salt. (Willstätter, *Z. physiol. Chem.*, 1914, **90**, 1; 1916–1917, **98**, 171.)

Solutions of alkali hæmatoporphyrin, prepared from dry blood or from the urine of a patient who had taken sulphonal, gave four distinct bands. The positions of the maximal points were $\lambda = 624\mu\mu$,

$\lambda = 574\mu\mu$, $\lambda = 544\mu\mu$, and $\lambda = 509\mu\mu$. The band in the violet, obtained by means of an isokol plate, is at $\lambda = 404\mu\mu$.

Pure hæmatoporphyrin hydrochloride in alkaline solution shows five bands, extending from red to blue, as follows: $\lambda = 614\mu\mu$, $\lambda = 563\mu\mu$, $\lambda = 535\mu\mu$, $\lambda = 501\mu\mu$, and $\lambda = 461\mu\mu$.

The violet band can be detected by using zircon light and a perortho plate, $\lambda = 388\mu\mu$.

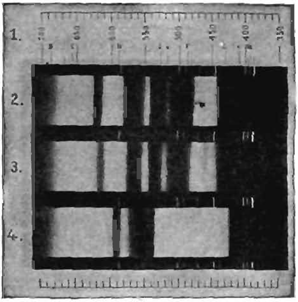


FIG. 20.

1. Scale of wave lengths.
2. 1.5 mgrm. hæmatoporphyrin in 10 c.c. 96 % alcohol.
3. 1.5 mgrm. hæmatoporphyrin in 20 c.c. 5 % NH_3.
4. 1.5 mgrm. hæmatoporphyrin in 10 c.c. 1 % H_2SO_4.

Fig. 20, taken from Rost, Franz, and Heise, shows the spectrum of hæmatoporphyrin hydrochloride in alcohol, ammonia, and sulphuric acid.

Crystallised pure haematoporphyrin in ethereal solution shows besides a weak thin band in the orange, 4 absorption bands, one very narrow, sharp and dark in orange towards red, one narrow and a little less dark at the beginning of the green, at which a very wide shadow filling the yellow region is shown and two strong bands in green and at passage of green into blue. The latter is widest and darkest. (Willstätter and Fischer, *Z. physiol. Chem.*, 1913, 87, 461.)

Hæmatoporphyrin, according to MacMunn (*J. Physiol.*, 1886, 8, 240; 1887, 8, 384) occurs in the integuments of certain molluscs and echinoderms. It is also found in normal human urine (Schulz,

Ergebn. f. Physiol., 1903, **2**, 159) (2–10 mg. per day) and in increased quantity in the urine in certain diseases, and especially in cases of sulphonal and trional poisoning. In the latter case it is found in the bile, liver and urine (Neubauer, *Arch. exp. Path.*, 1900, **43**, 541: Salkowski, *Z. physiol. Chem.*, 1891, **15**, 286).

The method recommended by Garrod (*J. Physiol.*, 1892, **13**, 598; 1894, **17**, 349) for its detection in urine is as follows: The earthy phosphates are precipitated from 500 c.c. urine by the addition of 100 c.c. of 10% alkali. The precipitate, which contains the hæmatoporphyrin, is washed and dissolved in an alcoholic solution of hydrochloric acid. The hæmatoporphyrin is again precipitated by the addition of ammonia, redissolved in acetic acid, filtered and extracted with chloroform. The chloroform is evaporated and the residue washed with alcohol and dissolved in acidified alcohol. This solution is examined spectroscopically. In view of the recent synthetic work of H. Fischer and others, it seems permissible to doubt whether some of the earlier observers really had in hand hæmatoporphyrin. Their spectroscopic results might well have been due to copro- and uroporphyrins.

Mesoporphyrin, $C_{34}H_{38}N_4O_4$

This substance was prepared by Nencki and Zaleski (*Ber.*, 1901, **34**, 997) by the reduction of hæmin.

Fischer and Meyer-Betz (*Z. physiol. Chem.*, 1912, **82**, 99; 1913, **84**, 278) give the following modification of Nencki and Zaleski's method: 5 grm. of haemin are warmed with 30 c.c. aqueous solution of hydriodic acid (sp. gr. 1.96) and 75 c.c. of glacial acetic acid in the water-bath until completely dissolved. With careful shaking this should be accomplished in ¼ hour. 10 c.c. water are now added, and during 10 minutes 3 grm. of phosphonium iodide in small lumps. The reaction has ended as soon as the initial dark reddish yellow fluid has turned bluish red, and no precipitate results on dilution of a sample with an equal volume of water. The fluid is now cooled, poured into 1 litre of water and treated with enough 10% caustic soda that it is just still acid with acetic. It is now filtered from the precipitate produced. For purification the material is dissolved in 300 c.c. of 1% caustic soda solution and made to 1 litre. The mesoporphyrin is again precipitated from the fluid by acetic acid. Further purification results on solution in sodium hydroxide solution

and precipitation as the sodium salt by adding a small excess of concentrated sodium hydroxide solution. By solution in 250 c.c. of boiling 2.5% hydrochloric acid it may be converted into a beautifully crystalline di-hydrochloride of mesoporphyrin, consisting of difficultly soluble needles. According to Willstätter and Fischer, crystalline mesoporphyrin may be obtained from the hydrochloride by neutralising the aqueous solution with ammonia and extracting with much ether. On evaporation of the ether the mesoporphyrin crystallises. (*Z. physiol. Chem.*, 1913, **87**, 442.)

Mesoporphyrin is very similar in properties to hæmatoporphyrin, but according to Nencki and Zaleski the ethers differ somewhat in solubility and melting point from those of hæmatoporphyrin.

In acid solution the spectrum has four bands, $\lambda = 608\mu\mu$, $\lambda = 589\mu\mu$, $\lambda = 567\mu\mu$, and $\lambda = 546\mu\mu$, the last being the most marked.

In the violet the maximal point of the band is $\lambda = 399\mu\mu$. In alkaline solution, using an isokol plate, seven bands have been distinguished: $\lambda = 633\mu\mu$, $\lambda = 615\mu\mu$, $\lambda = 583\mu\mu$, $\lambda = 560\mu\mu$, $\lambda = 535\mu\mu$, $\lambda = 501\mu\mu$, and $\lambda = 463\mu\mu$. With a zircon light and a perorthic plate a band, $\lambda = 402\mu\mu$ was obtained.

Hæmatoidin.—This iron-free derivative of hæmoglobin may occur as microscopic crystals in old blood extravasations within the body; for example, clots of blood within the brain or the contents of cysts in the liver. The crystals are orange-coloured, rhombic plates, soluble in chloroform. Hæmatoidin solutions show no spectrum, but the ultra-violet and blue rays are largely absorbed. It is generally considered that *hæmatoidin and bilirubin are identical from their crystalline characters*, and both give Gmelin's test—a play of colours when solutions are treated with nitric acid containing nitrous acid.

Phylloporphyrin, $C_{34}H_{38}O_2N_4$

This substance was obtained by Schunck and Marchlewski by the decomposition of chlorophyll. It is very similar in properties and in its spectrum to hæmatoporphyrin but differs from the latter substance in containing two oxygen atoms less. Nencki and Zaleski attempted to change hæmatoporphyrin directly into phylloporphyrin but were unsuccessful, obtaining instead mesoporphyrin. Both substances on reduction give the same decomposition product, hæmopyrrole and on oxidation the same hæmatinic acids.

Haemoporphyrin was obtained by Willstätter and Fischer (*Z. physiol. Chem.*, 1913, **87**, 492) by reduction of haematoporphyrin by heating a solution of 2 grm. in 50 c.c. of methyl-alcoholic potash and 100 c.c. of pyridine in an autoclave at 200° for 4 to 5 hours.

It is difficultly soluble in ether and absolute alcohol, insoluble in benzene and in chloroform. It is a dicarboxylic acid and forms a crystalline di-methyl ether and a di-hydrochloride. It has recently been found however by H. Fischer, Halburger and Hummel (*Z. physiol. Chem.*, 1930, **191**, 250) that haemoporphyrin prepared by Willstätter's method is a mixture of mesoporphyrin, deuteroporphyrin and two tetro methyl monoethyl dipropionic porphims derived from aetioporphyrin III.

Aetioporphyrin.—Fischer and Röse (*Z. physiol. Chem.*, 1916, **91**, 186) also found that by heating haematoporphyrin—or better its magnesium compound—with soda lime, it loses CO_2 and perhaps acetic acid and passes into aetioporphyrin.

Aetioporphyrin crystallises from ether in prisms, decomposing at 265°. It is difficultly soluble in alcohol and ether, but easily in acetone and hot glacial acetic acid. It is strongly basic and forms characteristic salts with picric and other acids.

Uroporphyrin H. Fischer (*Z. physiol. Chem.*, 1915, **95**, 34; 1916, **98**, 78) reported on a case of inborn porphyrinuria, and found besides a copro-porphyrin a special urine porphyrin, which he called *uroporphyrin*, whose spectrum was very like that of haemato-porphyrin but which differed in composition. It is not certain whether this is identical with porphyrin isolated from urine by previous observers or not.

Since this, uroporphyrin has been certainly proved by Löffler (L. *Biochem. Z.*, 1919, **98**, 105) in cases of idiopathic porphyrinuria, by Ellinger and Riesser (*Z. physiol. Chem.*, 1917, **98**, 1) in trional poisoning, in a case of severe nervous degeneration by Abderhalden (*Z. physiol. Chem.*, 1919, **106**, 178). Fischer isolates the uro-porphyrin by conversion into the beautifully crystalline methyl ester. 25 litres of the urine are acidified with 300 c.c. of glacial acetic acid, more if ammoniacal fermentation has set in. After standing overnight, the bulk of the clear fluid is poured off from the precipitate which has settled and this is filtered through keselguhr. After washing the residue with water, it is nearly but not quite dried, taken up in 40 c.c. of *N* caustic soda solution, made up to 1 litre and filtered.

The filtrate is acidified with acetic acid in slight excess *N* and the *precipitate filtered off under pressure through a hardened filter and well washed.* It is difficult to isolate the colouring matter from this precipitate owing to impurities. It is therefore suspended in 300 c.c. of methyl alcohol, cooled in air and saturated with hydrogen chloride. After standing 24 hours the fluid is diluted with an equal volume of methyl alcohol, filtered, and the filtrate poured into many times its volume of water. The solution is then carefully made alkaline with soda and the colouring matter extracted with chloroform. After evaporating the dried chloroform solution the crystalline residue is again dissolved in 70 c.c. chloroform, filtered, warmed and boiling methyl alcohol added. The ester of the uroporphyrin crystallises quickly. In the mother liquors is found the ester of the copro-porphyrin.

To get the free uro-porphyrin from the ester, the latter may be shaken for 1 hour with 60 times its weight of a solution of hydrogen bromide in glacial acetic acid. The clear solution is poured into a large excess of water and the porphyrin liberated by adding sodium acetate. It may be crystallised from a mixture of pyridine and alcohol. It forms fine red needles. According to Fischer the molecule contains 8COOH groups and the formula is $C_{40}H_{38}O_{16}N_4$. In *N*/10 potassium hydroxide solution the spectrum shows five absorption bands at 613–603, 577–565, 561–552, 542–532 and 511–496$\mu\mu$.

In 19 per cent hydrochloric acid there are three bands, 598–590, 576–571 and 558.5–544$\mu\mu$.

The methyl ester in chloroform solution shows a darkness at 587$\mu\mu$ and 4 bands at 628–620, 586–564, 541–526 and 513–485$\mu\mu$.

Coproporphyrin

This substance is present in quantity, together with uroporphyrin, in the excreta of patients with porphyrinuria. (*Z. physiol. Chem.*, 1915–16, **96**, 148; 1916, **97**, 148; 1916, **98**, 14.)

Post-mortem examination of the organs of a case of congenital porphyrinuria showed that this porphyrin is present in most of the organs. (Fischer, *Z. physiol. Chem.*, 1925, **150**, 40.) Bone marrow contains both copro- and uroporphyrin, the former in greater quantity. Bile contains considerable amounts of coproporphyrin; blood contains coproporphyrin, but no uroporphyrin. The intestine contains no uroporphyrin, but is rich in coproporphyrin. This

is in agreement with the findings in the fæces of the living subject. Apparently, bone marrow is the principal seat of porphyrin formation. Schumm, and also Papendiek, is of opinion that the coproporphyrin of normal urine should be also the coproporphyrin in disease. Fischer has found this coproporphyrin is present in small amount as a normal metabolic product in the fæces, especially after meat has been taken. According to Fischer and Hilger (*Z. physiol. Chem.*, 1925, **149**, 65), when dry uroporphyrin is heated at 180° in an atmosphere of nitrogen decarboxylation occurs and coproporphyrin is formed in 60% yield.

Coproporphyrin is isolated from fæces as follows: The dry faeces are thoroughly extracted first with ether and then with alcohol to remove fats, coprosterol, etc. The fat-free residue is then extracted three times with 1% bicarbonate of soda solution, which takes up the pigment, partly as porphyrin again, which gets oxidised during the prolonged filtration. The bicarbonate solution of the porphyrin is thus repeatedly filtered until a filtrate is obtained perfectly free from any suspended matter. The clear filtrate is acidified with acetic acid when the colouring matter is precipitated. This is now converted into the methyl ester by a method analogous to that used in the case of uroporphyrin and the ester extracted with chloroform. After evaporation of the chloroform the residue is recrystallised from a mixture of chloroform and alcohol (m. p. 249–250°).

In 1928 Hijmans van den Bergh and Mueller (*Arch. f. Verdauungs Krank.*, 1928, **42**, 306; also *K. Akad. van Wetenschaffen te Amsterdam*, 1929, **32**, 15) described a case of congenital porphyrinuria, which differed from all other cases described up to that time, in that the urine contained an ether-soluble coproporphyrin but no uroporphyrin. This coproporphyrin gave a methyl ester (m. p. 169°), and was isomeric with the coproporphyrin previously described. It was also found in the faeces of the patient. It proved identical with the coproporphyrin prepared synthetically by Fischer and related to aetioporphyrin III. The other ester is related to aetioporphyrin I. (See page 72.)

Constitution of the Porphyrins and of Haemin

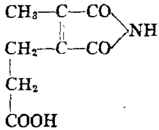
Although different haemoglobins appear to be present in the animal kingdom, the haemin obtained from them is generally regarded

as one and the same substance, though precise proof of this has not yet been forthcoming.

The constitution of the porphyrins has been arrived at in the usual manner—analytical breakdown into simple building stones followed by synthesis. Our knowledge of the building stones of the Haem molecule is largely due to the labours of Küster, Nencki, Piloty, Willstätter, Hans Fischer and their co-workers.

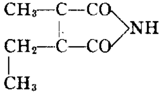
The most fruitful methods of breakdown have been (1) oxidation (Küster), (2) reduction with glacial acetic acid and hydriodic acid (Nencki) and (3) cleavage by means of sodium or potassium methoxide, which causes a reductive cleavage, much like that by hydriodic acid in glacial acetic acid, accompanied by alkylation. (Fischer and Röse, *Z. physiol. Chem.*, 1913, **87**, 381: 1913, **88**, 91; 1914, **91**, 184; *Ber.*, 1914, **47**, 781.)

Oxidation.—By the oxidation of haematin, haemin (Küster, *Ber.*, 1899, **32**, 677; *Z. physiol. Chem.*, 1899, **28**, 1; 1905, **44**, 391; 1909, **61**, 174; Annalen, 1901, **315**, 174), haemato-porphyrin (Küster, *loc. cit.*; *Z. physiol. Chem.*, 1909, **61**, 174), mesoporphyrin (Küster, *Ber.* 1912, **45**, 1945) and coproporphyrin (Fischer, *Z. physiol. Chem.*, 1916, **98**, 21) by means of chromic acid in glacial acetic acid Küster, and Willstätter and Asahina (Willstätter and Asahina, *Annalen*, 1910, **373**, 237) obtained as the first crystalline breakdown product the imide of the tribasichaematinic acid $C_8H_9O_4N$. Its constitution was proved by synthesis by Küster and Weller (*Ber.* 1914, **47**, 532; *Z. physiol. Chem.*, 1917, **99**, 229) who gave it the formula



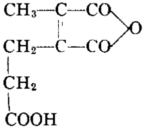
By oxidation of the di-methyl ester of haematin, Küster and Greiner (*Ber.* 1912, **45**, 2503; *Annalen*, 1906, **345**, 1) prepared the di-methyl ester of the haematinic acid. The acid crystallises from hot water in needles, and is soluble in the usual organic solvents. It melts at 113.5°–114.5°, and forms a series of crystalline salts.

If heated with alcoholic ammonia in a sealed tube at 130° or even by dry distillation, haematinic acid loses CO_2 and forms the imide of the dibasic haematinic acid



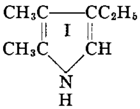
which also crystallises in needles and is easily soluble in ether and alcohol (Fischer, *Z. physiol. Chem.*, 1916, **98**, 21).

If the imide of tri-basic haematinic acid is hydrolysed by heating with 50% sulphuric acid or with baryta the anhydride of the tribasic acid (*Ber.*, 1914, **47**, 532; *Z. physiol. Chem.*, 1917, **99**, 229) is obtained.

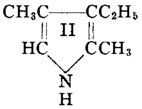


This anhydride crystallises in different forms and melts at 97–98°. It is most easily crystallised from water. It was prepared synthetically by Küster and Weller (*Ber.*, 1914, **47**, 532; *Z. physiol. Chem.*, 1917, **99**, 229). For a further account of the properties of these products the original papers must be consulted.

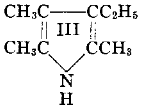
Reduction.—By drastic reduction by means of hydriodic acid in glacial acetic acid solution the following bases and acids have been obtained:



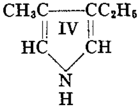
Haemopyrrole



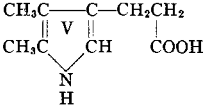
Cryptopyrrole



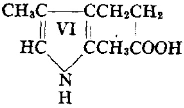
Phyllopyrrole



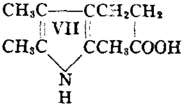
Opsopyrrole



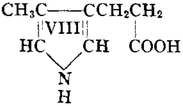
Haemopyrrol Carboxylic Acid



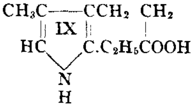
Cryptopyrrole Carboxylic Acid



Phyllopyrrole Carboxylic Acid



Opsopyrrole Carboxylic Acid

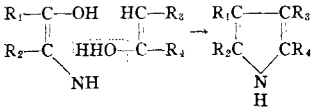


Xanthopyrrole Carboxylic Acid

A glance at the formulae will show that all the β-positions of the pyrrole nuclei have on the one side a CH_3 group and on the other a C_2H_5 or a $—CH_2CH_2COOH$ group. The α-positions are occupied by a hydrogen atom or a CH_3 group. Only the xanthopyrrol-carboxylic acid has a C_2H_5 group in the α-position.

The constitution of all these building stones has been shown by synthetic methods.

Amongst the methods of building up the pyrrole nucleus, one of the first, and perhaps the most useful, is that devised by Knorr (*Annalen*, 1886, **236**, 317), which consists in the condensation of a ketone with another α-aminoketone, according to the following scheme, in which the ketones are written in the enol form.



It is not always necessary to isolate the amine; it may be produced in the reaction mixture by reduction of a nitrosoacetone. In practice, ethyl aceto-acetate is most frequently the starting material. It is converted into a nitroso derivative by nitrous acid. This is reduced by zinc dust to the amine and simultaneously condensed with a second β-ketonic molecule.

According to A. Kirrmann (*Bull. Soc. Chim.*, 1930, [4], 57/58, 18) in the procedure in use in Fischer's laboratory the three opera-

tions are carried out without interruption and without isolation of intermediate products, in such a manner that one can in a few hours obtain any quantity of the pyrrole depending on the ethyl aceto-acetate taken.

Ethyl aceto-acetate condenses in this way with another molecule (of itself) to give 3:4-dimethyl-3:5-dicarbethoxy-pyrrole, and with acetyl-acetone to form 2:4-dimethyl-3-acetyl 5-carboxyethyl pyrrole.

Knorr and Hess (*Ber.*, 1911, **44**, 2758) obtained crypto-pyrrole from 2:4-dimethyl-3-acetyl-pyrrole by reduction by means of hydrazine and sodium ethoxide, thereby making the formula certain. In an analogous manner Piloty obtained haemopyrrole. The constitution of the four basic pyrroles and of acid VII was made certain by the work of H. Fischer and Bartholomäus. (*Ber.*, 1912, **45**, 466; 1912, **45**, 1315; 1912, **45**, 1894; 1913, **46**, 5112; *Z. physiol. Chem.*, 1912, **76**, 478; 1913, **83**, 50.)

The substituted pyrroles possess a great capacity for undergoing reactions, and are capable of numerous transformations, permitting of the lateral side chains being varied almost at will by transformation, addition or suppression of groupings.

Ethyl and methyl radicals may be introduced directly in place of a hydrogen atom of the nucleus by heating with an alkali alcoholate. The group preferably takes an α-position, *i. e.*, on the carbon next to the nitrogen.

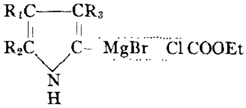
Another method is to introduce an aldehyde or a ketonic radical and reduce this by means of hydrazine and sodium ethoxide. Fischer and Zirweck (*Ber.*, 1922, **55**, 1912) found that the CHO group is easily introduced into an alkylated pyrrole by Gattermann's reaction with prussic acid, and an acetyl group by the Friedel and Crafts reaction between the pyrrole and acetyl chloride.

A serviceable method of eliminating a CH_3 in the α-position consists in halogenating the CH_3 group in the cold by means of sulphuryl-chloride (SO_2Cl_2), hydrolysing the CCl_3 group produced to COOH and eliminating CO_2 by heat.

In all these transformations, additions or suppressions of a CH_3 group, there appears to be a marked tendency to create or to preserve the grouping side by side the two methyl groups. For example, in the case of 3-methyl-4-ethyl-pyrrole, when a methyl group is introduced, with CHO as intermediary, it goes into position 2 and not position 5.

Fischer and Nenitzcocu (*Annalen*, 1924, **439**, 175) worked out the conditions for condensation of trimethyl pyrrole aldehyde with malonic acid and the elimination of a CO_2 molecule from the product, leading to an acrylic acid derivative, which on reduction gave phyllo-pyrrole-carboxylic acid VII. In an analogous manner crypto- and xantho- pyrrole carboxylic acids were built up.

Methods were also worked out for the oxidation of a CH_3 group to CHO and to COOH, and by the elimination of the latter de-methylation was possible. Fischer (working with Sturm, Friedrich and Lamatsch) in this way obtained opso-pyrrole and opso-pyrrole-carboxylic acid VIII. The introduction of a COOEt group is often useful to protect an α position. This can be effected by acting on the magnesium derivative of the pyrrole with ethyl-chloro-carbonate.



Halogens easily replace in the pyrrole nucleus an α hydrogen atom or a hydrogen atom in a CH_3 group situated in the α position. If it is desirable to protect a hydrogen atom in the α position against the action of bromine it may be replaced by a carbethoxyl group, and this afterwards again eliminated.

To-day the building stones of blood pigments are all obtainable synthetically in almost any quantity that may be desired, and this material led to the synthesis in Fischer's laboratory of numerous porphyrines and finally of haemin itself.

The yields of products obtained by reduction and by the action of alkali-alcoholates led with some certainty to the conclusion that the haemin molecule contains 4 pyrrole nuclei—two basic and two acidic.

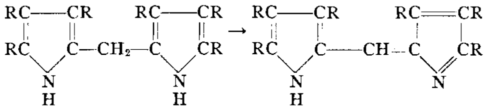
The molecular weight, deduced from the rise of boiling point of pyridine solutions, was found by Fischer and Hahn (*Ber.*, 1913, **46**, 2308) to be about 600.

Possible structures for haemin were proposed by Küster, Willstätter and Fischer as a result of the degradation of the porphyrines and of haemin. Fischer then began his investigations on the synthesis of porphyrins, and during the past three or four years

extraordinary progress has been made. Fischer has summarised his results from time to time (*Ber.*, 1927, **60**, 2611: *Naturwiss.*, 1929, **17**, 611). Also A. Kirrmann (*Bull. Soc. Chim.*, 1930, [4], **47–48**, 913). The writer has made much use of these papers in preparing this article.

The first step in the synthesis of the porphyrins was the investigation of the modes of formation and properties of the dipyrryl methenes.

The dipyrryl methenes are basic substances usually isolated in the form of their mono-hydro-bromides. The dipyrryl methanes, originally discovered by Colaccici, are easily converted into methenes by oxidation, *e. g.*

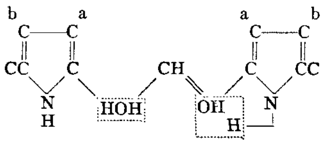


Symmetrical methanes may be obtained by condensation of two molecules of a pyrrole containing a hydrogen atom in α position, with formaldehyde, or by boiling a pyrrole, with a $—CH_2Br$ group in the α-position, in a methyl alcohol solution.

Unsymmetrical methanes may be prepared by condensing a pyrrole containing a $—CH_2Br$ group in α-position with another pyrrole with a hydrogen atom in the α-position.

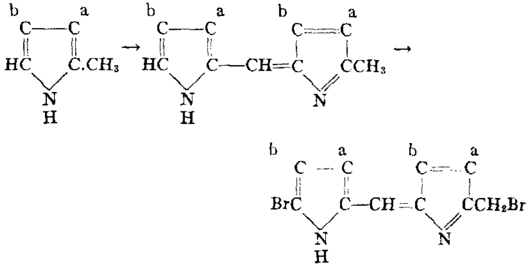
These methanes on oxidation give the corresponding methenes.

Symmetrical methenes may also be obtained directly by condensation of pyrroles with formic acid analogously to the formation of methanes by use of formaldehyde.



For the production of unsymmetrical methenes perhaps the most important method is the condensation of a pyrrole, containing an aldehyde group in the α-position, with a pyrrole containing an α-hydrogen.

Unsymmetrical methenes may also be prepared directly, starting from one pyrrole only. The two nuclei of the methene in that case differ not in nature but in the order of the substituents from α. Thus by the more or less energetic action of bromine on some tri-substituted pyrroles mono- and di-brom-methenes are formed.



Synthesis of Porphyrins.—In the methenes two pyrrolic nuclei being condensed together, it is only necessary to condense two methenes in a similar manner to produce a porphyrine. It might be predicted that this second condensation would happen analogously to the first. This is not the case, however, for the actual synthetic methods employed are much more drastic in practice.

Amongst the different methods employed one only is analogous to the preceding, it consists in boiling a pyrrole containing two α hydrogens with formic acid, *e. g.* opso-pyrrole with formic acid (Fischer and Treibs, *Annalen*, 1926, **450**, 132).

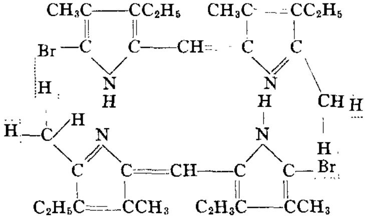
The other methods are all based on the use of di-pyrryl-methenes carrying one or two substituent carbon groups in an α-position and necessitate no external carbon material.

One of the first syntheses to be effected was by the action of concentrated sulphuric acid on a bromo-methene hydro-bromide, obtained by brominating crypto-pyrrole in acetic acid solution. This method gave an aetio-porphyrin spectroscopically identical with Willstätter's aetio-porphyrin from natural sources. (Fischer and T. Klarer, *Annalen*, 1926, **450**, 181.)

Another procedure consists in dissolving a di-pyrryl methane with a COOEt group in an α position in formic acid and passing a current

of air through this solution at 40°C. (Fischer, P. Halbig and Walach, *Annalen*, 1927, **452**, 268.) This method was usefully applied in the synthesis of a uroporphyrin, for which it was the only one possible, since higher temperatures decompose the product to a copro-porphyrin.

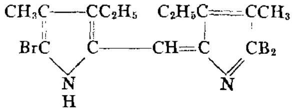
The most general and on the whole the most satisfactory method consists in fusing a methene or two chosen methenes with several times their weight of succinic acid for several hours at 200° or thereabouts. Other acids such as methyl-succinic acid and benzoic acid, may also be used. Thus for example a molecule of aetio-porphyrin is obtained at the expense of two molecules of bromo-methene from crypto-pyrrole.



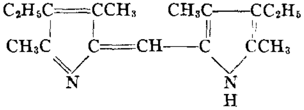
The reaction is pretty complex, however, and illustrates the remarkable tendency for porphyrins to form. The methenes employed (or better their hydro-bromides which give better yields) carry at the α position different combinations two by two of the following groups: H.Br.CH_3.CH_2Br. The object is to form between the methenes the bridges —CH=, of which the atoms are furnished by a CH_3 or a CH_2Br group of a methene. On the second molecule of methene there must be an atom of Hydrogen or Bromine facing these groups.

Different conditions of symmetry govern the formation of porphyrines. Thus a methene, of which one only of the α positions is occupied by a CH_3 or CH_2Br, can form a porphyrine by the condensation of two identical molecules. In general one can only obtain in this way a porphyrin whose formula possesses a centre of symmetry. A mixture of two methenes A and B would give rise to the formation of three porphyrines, A + A, B + B and A + B.

The last would be unsymmetric, but it is only possible to separate the others in particular cases. Methenes in which the two α positions are both occupied—it may be by H (or Br) or it may be by CH_3 (or —CH_2Br)—cannot form porphyrin alone, but a mixture of the two kinds gives excellent results. For example:



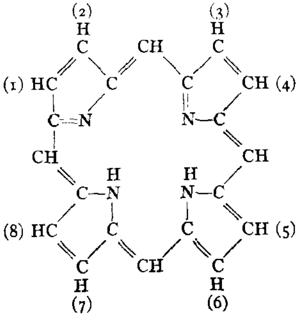
and



give aetioporphyrin.

The yields by fusion with succinic acid vary considerably, but mostly vary round 20%.

From the analytical cleavage products and the synthetic methods discovered by Fischer it would appear that the numerous porphyrines are derived from a, as yet, hypothetical simple porphyrin consisting of 4 pyrrolic nuclei joined by four —CH=bridges at the α positions.



This would theoretically yield 4 isomeric aetio-porphyrins:

(1) 1:3:5:7 tetramethyl 2:4:6:8 tetraethyl porphyrin m. p. 400°–405°

(2) 1:4:5:8 tetramethyl 2:3:6:7 tetraethyl porphyrin m. p. 365°

(3) 1:3:5:8 tetramethyl 2:4:6:7 tetraethyl porphyrin m. p. 360°

(4) 1:4:6:7 tetramethyl 2:3:5:8 tetraethyl porphyrin m. p. 358°

These isomers have all been synthesised, in every case by two methods, and have proved different. (Fischer and Stengler, *Annalen*, 1927, **459**, 53.) Aetio-porphyrins I and III are the isomers related to natural porphyrins.

These results were fully confirmed by the synthesis of the copro-porphyrins, theoretically derived from the aetio-porphyrin by replacement of the 4 ethyl groups by 4 propionic acid residues—$—CH_2CH_2COOH$. These copro-porphyrins readily form esters in the usual way. These esters possess sharp melting points and consequently the copro-porphyrins are more easily identified and differentiated than the aetio-porphyrins. All four isomers have been synthesised. They are spectroscopically identical, or very nearly so, but the melting points of their esters differ, and mixed melting points of mixtures of the different esters show marked depression.

Copro-porphyrin I, *i. e.* 1:3:5:7-tetramethyl, 2:4:6:8-tetra-propionic acid porphyrin, is the copro-porphyrin isolated from faeces and from normal and pathological urine (cases of poisoning with lead, sulphonal etc.) and from yeast.

Copro-porphyrin III, 1:3:5:8, tetramethyl 2:4:6:7, tetra-propionic acid porphyrin is identical with the copro-porphyrin isolated by Hjimans van den Bergh from urine and faeces of a case of porphyrinuria. (*K. Akad. v. Wetenschaffen, Amsterdam*, 1929, **32**, 15.)

The meso-porphyrins, derived from the four aetio-porphyrins by replacing two ethyl groups by two propionic acid residues are theoretically 15 in number. Nine of these have been already synthesised, amongst them the natural meso-porphyrin got from haemin. This is 1:3:5:8 tetra-methyl, 2:4 di-ethyl, 6:7 di-propionic acid porphyrin. The most characteristic property of this substance is the slight solubility of the sodium salt.

The uro-porphyrin found in urine, particularly pathological urine, is also found in the form of a copper compound—turacin—in the colouring matter of birds' feathers, and is the most highly oxygenated of the porphyrins. Theoretically, there should be four

isomers, corresponding to the four copro-porphyrins, from which they differ in having a methyl malonic-acid residue $-CH_2CH(COOH)_2$ in place of the propionic acid residues.

They are rather unstable, and only one, an isomer of the natural substance, has been synthesised. (Fischer and H. Heisel, *Annalen*, 1927, **457**, 83.)

Synthesis of Haemin.—The synthesis of haemin had to be effected by introducing the unsaturated group $-CH{=}CH_2$ into an already synthesised porphyrin, since the attempts to synthesise methenes with unsaturated side chains proved unsuccessful.

In the following table (after Fischer) is given a list of the chief blood pigment porphyrins:

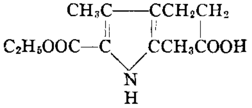
BLOOD PIGMENT-PORPHYRINS RELATED TO AETIO-PORPHYRIN III—AFTER FISCHERA

Name	Formula	Occurrence in nature	Preparation	Side chains
Proto-porphyrin........	$C_{34}H_{34}N_4O_4$	In spreckled egg-shells (oo-porphyrin); By blood putrefaction, Kammerer's porphyrin; in yeast etc.	Formic acid and iron	4 methyl, 2 vinyl groups, 2 propionic acid residues.
Meso-porphyrin........	$C_{34}H_{38}N_4O_4$	....	Acetic acid and HI.	Instead of vinyl, ethyl group in above.
Haemato-porphyrin.....	$C_{34}H_{38}N_4O_6$	....	Acetic acid and HBr.	Instead of vinyl group two α oxy-ethy residues − CHONCN₂
Aetio-porphyrin........	$C_{32}H_{38}N_4$	....	Pyroreaction.	$4CH_3$ and $4C_2H_5$ groups.
Deutero-porphyrin.....	$C_{30}H_{30}N_4O_4$	Protracted blood putrefaction.	....	Substitution of vinyl groups by hydrogen.
Copro-porphyrin III	$C_{36}H_{38}N_4O_8$	Isolated from case of porphyrinuria by van den Bergh.	Ester method methyl-ester m. p. 169.	4 methyl and 4 propionic residues.
NATURAL PORPHYRIN RELATED TO AETIO-PORPHRYRIN I				
Uro-porphyrin I........	$C_{40}H_{38}N_4O_{16}$	In urine with congenital and acute porphyrinuria copper salt = Turacin.	By ester method. Methyl-ester m. p. = 293°.	4 methyl and 4 methyl malonic acid residues.
Copro-porphyrin I......	$C_{36}H_{38}N_4O_8$	In yeast, in normal urine and stools in poisoning by lead and sulphonal etc.	By ester method. Methyl-ester m. p. 250°	

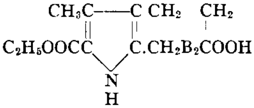
The synthesis of haemin was effected as follows:

4:5 di-methyl pyrrole was condensed with 2:4 di-methyl 5-formyl pyrrole, to form the methene (4:5-di-methyl pyrryl)—(5′3′ dimethyl pyrrolenyl) 22″ methene hydro-bromide.

By bromination of carbethoxylated crypto-pyrrole carboxylic acid was obtained. This, on boiling with water, condensed to the methane —bis (3 propionic acid, 4 methyl, 5 carbethoxyl pyrryl) methane. The—$COOC_2H_5$ groups in this were readily saponified to COOH

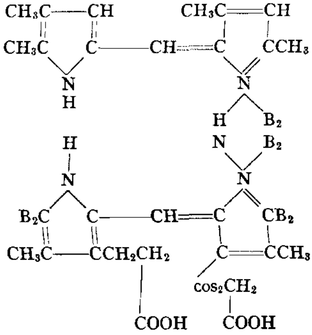


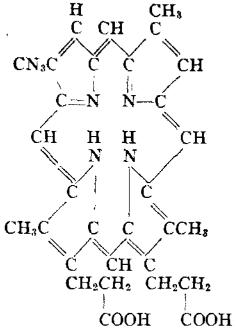
the bromo derivative



groups. This methane on bromination lost CO_2 and was oxidised to the methene (5 bromo, 4 methyl, 3 propionic pyrrole)—(5[1] bromo, 4[1] methyl, 3[1] propionic acid pyrrolenyl) methene hydrobromide.

When these two methenes were fused with excess of benzoic acid they condensed to form deutero-porphyrin.





Deuteroporphyrin

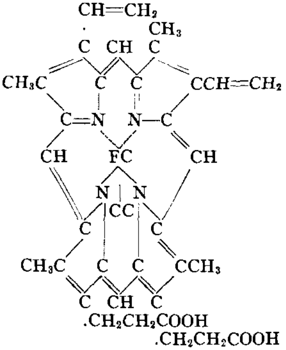
This deutero-porphyrin proved rather inert chemically and it was therefore converted, by the introduction of the iron complex, into deutero-haemin. This was acetylated by means of acetic anhydride and stannic chloride, by which the hydrogen atoms 2 and 4 were replaced by acetyl groups forming di-acetyl deutero-haemin. The iron complex was now removed and the di-acetyl-deutero-porphyrin obtained. On reduction by means of alcoholic potash, the acetyl groups —CO CH_3 were reduced to methyl-carbinol groups —CHOH CH_3, and haemato-porphyrin was obtained. This, therefore, has the constitution 1·3:5·8 tetra-methyl, 2:4-di-α-hydroxy-ethyl, 6:7-di-propionic acid porphyrin. This substance proved to be identical in every respect with ordinary haemato-porphyrin, the oldest known porphyrin.

On heating the haemato-porphyrin to a high temperature two molecules of water were eliminated, and the —$CHOHCH_3$ groups passed into vinyl groups —CH=CH_2. The porphyrin so obtained was identical with proto-porphyrin (oo-porphyrin and Kammer's porphyrin were shown to be identical and re-named proto-porphyrin by Fischer and F. Linder (*Z. physiol. Chem.*, 1925, **142**, 141).

The methyl ester of this proto-porphyrin gave no depression in a mixed melting determination with a proto-porphyrin methyl ester prepared from haemin (*Annalen*, 1929, **468**, 98).

This proto-porphyrin on combination with iron yielded crystals of haemin identical in every respect with the ordinary haemin prepared from haemaglobin.

The formula of baemin is therefore:



There can be no doubt from Fischer's work that the carbon structure and the arrangement of the substituent groups is correct. The only part that requires some further clearing up is the exact mode of union of the iron complex.

Hæmocyanin was first investigated by Fredericq and later by Halliburton. It is a copper-containing protein found in the blood of various invertebrates, such as the *Octopus vulgaris*, the lobster, the edible snail, crabs and crayfish. It appears to play the same part as hæmoglobin in the blood of mammals. When reduced the pigment is colourless, but when associated with oxygen becomes pale blue.

Hæmocyanin has been obtained in a crystalline form by Henze (*Z. physiol. Chem.*, 1901, **33**, 370, and Dhéré, *Compt. rend.*, 1908, **146**, 784). According to Henze, it contains 0.38% of copper. It is generally described as possessing no characteristic spectrum, but Dhéré states that in a stratum 3 mm. tbick containing 7.59 grm. per litre, a band in the ultra-violet exists ($\lambda = 292.2\mu\mu$–$\lambda = 262.8\mu\mu$). On increasing the thickness of the layer a fresh band appears, and with a stratum 10 mm. thick it extends to $\lambda = 364\mu\mu$–$\lambda = 328\mu\mu$.

This band is characteristic, but the more refrangible band is common to all albuminous substances.

Turacin.—This is another copper-containing pigment found in the feathers of musophagidæ (plantain-eaters). Turacin, according to A. H. Church, is turacoporphyrin and contains 7.1% of copper. P. P. Laidlaw (*J. Physiol.*, 1904, **31**, 469) succeeded in making from hæmatoporphyrin, prepared from hæmin by Nencki and Sieber's method, a copper hæmatoporphyrin indistinguishable from the naturally occurring pigments. For its formation all that is required is to add to a solution of pure hæmatoporphyrin in ammonia some cupra-ammonium solution and boil; turacin is formed at once.

Turacin recalls in its spectrum the blood-colouring matter. It has never been crystallised. The spectrum is two-banded—λ = 555–575$\mu\mu$, and λ = 520–λ = 545$\mu\mu$. H. Fischer regards it as a uroporphyrin derivative. For a further account see D. Keilin *Proc. Roy. Soc.*, 1926, **13**, 100, 129.

Quantitative Estimation of Hæmoglobin.—For this a great many methods have been devised, based upon the various chemical and physical properties of blood-pigment. We give only a few of those which are most commonly used.

Chemical Methods

1. The amount of oxygen in a sample of blood may be determined by expelling the entire gas. This can be done with the blood-gas pump (*J. Physiol.*, 1910, **40**, 374) or, for very small quantities of blood, by the ferricyanide of potassium method of Haldane, with the blood-gas apparatus suggested by Barcroft (*J. Physiol.*, 1908, **37**, 12; 1910, 429; 1911, **42**, 512). On the assumption that 1.34 c.c. of oxygen at 0° and 760 mm. are combined with 1 grm. of hæmoglobin, the percentage of this can be calculated.

2. By the determination of iron contained in the ash of blood. As it is known that pure hæmoglobin contains 0.336% of iron, and as all the iron in the blood exists in hæmoglobin, we may calculate the amount of this pigment, if the iron in the ash of a known weight or volume of blood is estimated.

About 100 grm. are evaporated to dryness in a platinum basin and then ignited. The incineration of blood is somewhat troublesome. The method in use in Bunge's laboratory is to mix the organic sub-

stance with sodium carbonate solution until distinctly alkaline, and then add further an equal amount of this solution. If the material is alkaline to begin with, about 0.5 to 1 grm. of sodium carbonate is added to every 100 grm. The mixture is placed in a platinum dish and evaporated to dryness in a water-bath, with constant stirring. It is finally dried at 120° and then carefully incinerated over a Bunsen flame. It is essential that the carbon should be burnt off at the *lowest possible temperature*. If the carbon is not completely burnt away, iron will remain which cannot be removed from carbon by boiling with acids, but if the ash is heated to too high a temperature the oxide of iron becomes very insoluble. It is better to burn off the bulk of the carbon at a low red heat, then extract with strong hydrochloric acid, and to filter through an iron-free filter. The filter-paper and unburnt carbon are then completely burnt. A. Neumann recommends, instead of incinerating in the dry way, to oxidise the organic matter by gentle warming with a mixture of equal volume of concentrated nitric and sulphuric acids. This may be done in a long-necked flask closed with a loose stopper or funnel, as is adopted in the determination of nitrogen by Kjeldahl's method. The authors prefer to oxidise the organic matter with excess of 100% volume hydrogen peroxide (Merck, *Proc. Physiol. Soc.*, 1906, **35**, 32). The action on substances like hæmin is often intense, much heat being evolved and the liquid raised to the boiling-point. For the determination of the iron in the ash, any gravimetric method, having due regard to the composition of the ash, may be employed. The most suitable method is that of titration with titanium chloride devised by Knecht and Hibbert (*Ber.*, 1903, **36**, 1551).

As it is not always possible to obtain a sufficiency of material for the above procedure, various methods have been suggested by which the iron can be determined colorimetrically in minute quantities of blood.

The best known is that of A. Jolles. (*Pflüger's Arch. Physiol.*, 1897, **65**, 579; *Deutsches med. Wochenschr.*, 1898, 104; *Berl. klin. Wochenschr.*, 1899, 965; *Münch. med. Wochenschr.*, 1901; see also F. Müller, *Virch. Arch.*, 1901, **164**, 436). A known amount (0.05 c.c.) of blood is incinerated in a porcelain or platinum dish, and the remaining oxide of iron dissolved by fusion with potassium hydrogen sulphate. The contents are then dissolved in water and treated with potassium or ammonium thiocyanate. The quantity of iron is

determined colorimetrically in the colorimeter. As 0.05 c.c. of blood contains only about 0.00002 grm. of iron, it is not possible to determine this with such accuracy as to be of much real use for ascertaining the amount of hæmoglobin, for, in addition to the inherent errors of the manipulations of small quantities of blood the colour of the ferric thiocyanate is not stable.

Colorimetric Methods.—In 1865 Hoppe-Seyler prepared a crystallised oxyhæmoglobin solution of known percentage strength. This is placed in a hæmatinometer (page 93). A weighed amount of blood made feebly alkaline with potassium hydroxide is placed in a second hæmatinometer and distilled water added from a burette, and the whole mixed with a long fish-bone until the tints examined against white paper are equal. The percentage by weight of oxyhæmoglobin in the tested blood is given by the following formula. If the weight in grm. of hæmoglobin in 100 grm. of the standard solution = h, the weight of blood examined = b and the grm. of water added to produce equality = w, then the blood solution contains

$$\frac{(b + w)h}{100} \text{ grm. } HbO_2$$

and the weight of blood taken contains

$$\frac{(b + w)h}{wb}\% \text{ by weight of } HbO_2.$$

Rajewsky has shown that this method gives excellent results, but is open to the objection that the oxyhæmoglobin solutions are not stable. He used coloured solutions of picrocarmine standardised against pure oxyhæmoglobin. These picrocarmine standards, however, also undergo a change in colour within a few months.

The Gowers-Haldane *Hæmoglobinometer* (Fig. 21) (*J. Physiol.*, 1900–1, **26,** 496). The standard tube is a 1% solution of defibrinated ox-blood saturated with coal gas, sealed up in an atmosphere of coal gas. The mean percentage oxygen capacity of this blood, as determined with Haldane's ferricyanide method, was 18.5%. The percentage of hæmoglobin corresponding to 18.5% oxygen capacity is 13.8, and the percentage carbon monoxide capacity is identical, 18.5. A tube similar in bore to the standard is graduated, and if 20 cb. mm. of normal human or other blood, saturated with carbon monoxide and diluted with water to the mark 100, exactly corre-

sponds with the standard, such a blood has a percentage oxygen capacity of 18.5 c.c. = 13.8% hæmoglobin.

Place distilled water saturated with coal gas or carbon monoxide in the graduated tube to mark 20. Suck up exactly 20 cb. mm. of blood in the capillary pipette. This is best done by exceeding the mark, wiping the end of pipette clean and gently dabbing the end on the back of the hand until the blood stands at 20. Now eject this to the bottom of the water, and with careful suction wash out the pipette with the water above the drop of blood. Now carefully

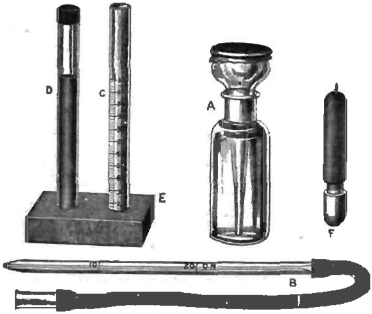


FIG. 21.—A, Bottle with pipette stopper for holding the diluting solution. B, Capillary pipette for measuring the blood. C, Graduated tube for measuring the amount of hæmoglobin. D, Standard tube. E, Support for D and C. F, Puncturing needle.

add distilled water saturated with coal gas or carbon monoxide drop by drop, mixing until the tint appears equal to the standard. Wait a minute, compare the tints again, holding the tubes against the light and frequently changing the tubes from side to side. Add a drop or a few drops more of water until the tints are just unequal; read again, and take the mean of the first and second readings as correct. The average percentage of hemoglobin of woman's blood is 11%, of children 13%, below the average of adult men.

Therefore, in calculating proportion of hæmoglobin in the blood of women and as percentages of the average normal proportion, it is necessary to add about 1/8 for women and 1/7 for children to the percentage found by the hæmoglobinometer with the standard solu-

tion described above. The maximum error of this instrument is ±0.8%. Any kind of light, that of the sun, electric light, gas or candle, can be employed in estimating the tint. The standard, kept in the dark, remains constant for years.

Sahli's hæmoglobinometer is similar in principle, and also in method of use. The standard is a solution of acid hæmatin. The blood examined is treated with 10 times its volume of $N/10$ hydrochloric acid to convert the hæmoglobin into acid hæmatin, and subsequently diluting to match the standard.

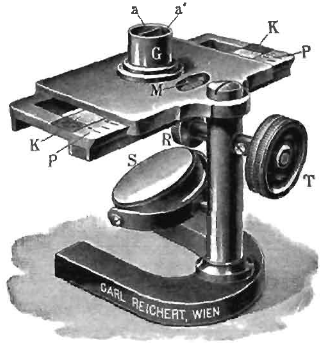


Fig. 22.—Hæmometer of v. Fleischl.

The *hæmometer* of von Fleischl.[1] (Fig. 22.) This apparatus consists of a stand something like the dissecting microscope. The mirror S is a plain white reflecting surface. Under the stage, M, is a slot carrying a wedge, K, of coloured glass (gold-purpur), the colour of which shades from deep red to almost pure white. By means of a milled wheel, T, this slides under the platform. A small metallic cell, G, divided by a vertical partition, stands on the stage, so that one compartment, a', filled exactly with distilled water, lies over the sliding wedge. The other compartment (a) is filled to a

[1] Miescher has modified this hæmometer, which possesses several improvements, in the method of mixing the blood and water, and in devices to vary the actual thickness of the stratum of blood or water which is examined. *This instrument—the hæmometer of Fleischl-Miescher*—is supplied by H. C. Reichert of Vienna. Full instructions are given for the use of the instrument.

quarter with distilled water. A capillary pipette with a metal handle is filled by dipping one end into a drop of blood, the ends wiped clean, so that the blood is just flush with the surface at each end; it must not present a concave or convex surface. The capillary is inserted into the partly filled compartment and moved about vigouously until all the blood is out. Finally the capillary is removed, its contents ejected into the blood and water, thoroughly mixed by stirring with the metal handle of pipette, and the compartment filled up exactly with water. The instrument is generally made for use with artificial and not sunlight. It is necessary to sit sideways, so that the wedge moves toward and away from the observer. By turning the milled screw the wedge is so moved as to match the blood exactly, and on the scale of the metal frame holding the wedge the hæmoglobin percentage is directly read off. In use, as little light as possible should be reflected through the cell from the surface of the plaster mirror. A short black tube of paper over the cell, the use of one eye, and quick judgment in matching the tints, are details for the successful use of the hæmometer. Its error is about 5–10%.[1]

Oliver's Hæmoglobinometer (*Lancet*, 1896, p. 1699).—In this instrument a specimen of blood suitably diluted in a shallow white palette is compared with a number of standards prepared by the use of Lovibond's tintometer glasses.

The Hæmoglobinometer of Dare (*Philadelphia Med. J.*, 1900, Sept. 22).—In this instrument the blood is examined without any dilution. The apparatus consists of a standardised disc of coloured glass mounted upon a disc of milk-white glass. The tint of this varies, and the depth of colour is standardised in percentages of hæmoglobin, and the disc can be rotated so as to match the sample of blood. On looking down a short brass tube carrying a lens, two circular holes can be seen side by side, illuminated with a candle flame. Behind one hole the coloured disc can be rotated, and behind the other is placed a special blood capillary. This consists of two small flat plates of glass, one clear, the other milk-white. Blood is received into the capillary space, and the capillary at once fixed in the instrument, so that on looking down the lens-tube two circular coloured discs are seen. That of the blood is matched by rotating the standard, and the value read off directly on the graduations of

[1] For the comparative value obtained with the hæmoglobinometers of Gowers-Haldane, v. Fleischl and Oliver, see Buckmaster, "*Morphology of Blood*," 1906 (p. 204).

the instrument. The observation must be carried out quickly before the blood coagulates or any deposition of corpuscles takes place in the capillary space.

A more accurate method, using a Duboscq or other suitable form of colorimeter, has been described by Newcomer (*J. Biol. Chem.*, 1918, **37**, 468; 1923, **55**, 569). The colour of haematin hydrochloride is matched in the colorimeter against a special coloured glass 1 mm. in thickness. A modification in which a haematin standard is used has also been described by E. H. Terrill (*J. Biol. Chem.*, 1923, **53**, 179).

In *Henocque's hæmoglobinometer* (Fig. 23) the hæmoglobin is estimated by observation of the absorption bands of oxyhæmoglobin.

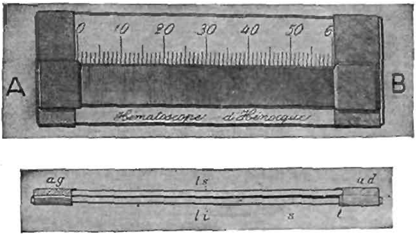


FIG. 23.

The apparatus consists of a broad glass plate on which is ruled a millimetre scale, 0–60. The plate has two brass ends, with slits A and B, into which can be dropped two glass plates so mounted as to enclose between their faces a wedge-shaped space; the apex of the wedge *ag* lies opposite the 0° of the scale, and at the 60 mark the distance between the plates at *ad* is 0.3 mm., so that as one passes along the scale the distance between the plates increases by 0.005 mm. for each division. Thus at scale mark 20 the distance will be $0.005 \times 20 = 0.1$ mm.

A direct vision spectroscope with a scale of wave-lengths is used to examine the blood.

The wedge-shaped space is filled with undiluted blood by capillarity, and the wedge placed in the brass slits of the scale-plate and the spectrum observed with daylight and the slit of the spectroscope set at 1 mm. The spectroscope is moved along against the blood

until the two bands of oxyhæmoglobin of $\lambda = 594\mu\mu$, $\lambda = 570\mu\mu$, and $\lambda = 536\mu\mu$–$\lambda = 530\mu\mu$ appear equally wide. As a control, the position is also read in which the two bands just appear and the point at which they just coalesce into one band. The concentration of oxyhæmoglobin varies with the thickness of the strata. Tables are constructed and supplied with the instrument, giving the percentage of hæmoglobin in terms of the millimetre scale. The graduation of the scale is based on the iron content of the blood and its oxygen capacity.

The spectrophotometer affords the most exact means of determining the amount of hæmoglobin or its derivatives in any fluid.

The ratio of the concentration to the extinction coefficient for a given colouring matter, provided the extinction coefficient is always determined for the same region of the spectrum, is a constant—which Vierordt called the "absorption relation." *Definition of the Term Absorption-relation.*—The more concentrated a coloured liquid is the greater is its absorptive power, and the less thick is the stratum of liquid required to reduce the intensity of light traversing it to $1/10$ of its original value. From the definition of the extinction coefficient, it therefore follows that the greater the concentration of solution the greater will be the extinction coefficient. If C is the concentration, *i.e.*, the mass of colouring matter in 1 c.c. of solution, and ϵ is the extinction coefficient for the selected region of the spectrum, $C/\epsilon = A$. If C', C'', etc., are the concentrations of different solutions of a colouring matter, and ϵ', ϵ'', etc., the corresponding coefficients for the same spectral region, then $C/\epsilon = C'/\epsilon' = C''/\epsilon''$ etc. $= A$. The procedure for the quantitative estimation of hæmoglobin and its derivatives is as follows: Let us take as an example oxyhæmoglobin. Human blood (20 c.c. is amply sufficient) is diluted 100 to 120 times its volume with 0.1% sodium hydroxide solution. The extinction coefficients of this solution are then determined for the regions $\lambda = 534.0\mu\mu$–$\lambda = 542.0\mu\mu$ and $\lambda = 556.5\mu\mu$–$\lambda = 564.5\mu\mu$,[1] and Hüfner's quotient of ϵ'/ϵ thus determined should be 1.578 with a possible error of 2.5%. The value or this quotient itself is a proof of the purity of oxyhæmoglobin, of that in the solution only oxyhæmoglobin is present.

The absorption relations of oxyhæmoglobin, given by Hüfner, in the two regions of the spectrum are given below.

[1] Instead of these regions, $\lambda = 531.5\mu\mu$ $\lambda = 542.5\mu\mu$ and $\lambda = 554\mu\mu$–$565\mu\mu$ can be used.

For oxyhæmoglobin, $A_1 \pm 0.001312$ and $A = 0.002070$. Supposing the values for ϵ' and ϵ for a given solution under examination are 0.895 and 0.565, then $\epsilon'/\epsilon = 0.895/0.565 = 1.58$; this indicates that only oxyhæmoglobin is present. The concentration will be given by,

$$C = \epsilon.'A' = 0.895 \times 0.001312 = 0.00117$$

or

$$C = \epsilon.A = 0.565 \times 0.00207 = 0.00117,$$

that is to say, 1 c.c. of the solution under examination contains 0.00117 grm. of oxyhæmoglobin.

If the two values are not quite the same, it is perhaps not altogether justifiable to take the mean. The reading for ϵ' is generally more exact than for ϵ. If the absolute concentration is not required, but the *relative* value for the two solutions, then the extinction coefficients are determined for these two solutions in the two spectral regions, and as the extinction coefficients are proportional to the concentrations, the relative concentrations are found.

The blood-colouring matter must be entirely in the state of HbO_2 or HbCO or Hb or met.-Hb. In the following Table 4 (Hüfner) *The Absorption Relations of Hæmoglobin and its Various Derivatives* are given, and these must be known in order to carry out determinations of the concentration of solutions.

TABLE 4

Colouring matter	Absorption relations $\lambda = 531.5-\lambda = 542.5$	$\lambda = 554-\lambda = 565$
HbO_2	A^1 0.001312	A = 0.002070
Met.-Hb	A^1 0.001754	A = 0.002078
Hb-CO	A^1 0.001516	A = 0.001829
Hb (red)	A^1 0.001778	A = 0.001354

Butterfield (*Z. physiol. Chem.*, 1909, **62**, 173) gives for HbO_2, A^1 for region 533.5-542 as 0.00118, and for A in the region $\lambda = 556.1-564.6$ as 0.00187. More recently W. H. Walker and C. S. Williamson (*J. Biol. Chem.*, 1920, **41**, 75) have redetermined the optical constant of haemoglobin. They give the absorption coefficients for dog, ox, cat, chicken, guinea-pig, rat, sheep, horse, pig and man. Their figures for man are practically identical with the above figures of Butterfield. Their values for different animals show slight

variation, but they do not consider the differences sufficiently marked to be of practical use in determining the species of animal from which blood is derived.

It is also possible to determine quantitatively the proportion of two colouring matters, provided they do not act upon one another in solution. Vierordt has given the proof that in a solution which contains two or more colouring matters, such colouring matter individually absorbs light in proportion to its concentration and its absorption relation, so that the extinction coefficient of a mixture is the sum of the extinction coefficients of its constituents; so that if $\epsilon_{1\cdot 2}$ is the extinction coefficient of the mixture and ϵ_1 and ϵ_2 those of the constituents, then

$$\epsilon_{1\cdot 2} = \epsilon_1 + \epsilon_2 \qquad (1)$$

and let x be the unknown concentration of a solution of one colouring matter and ϵ_1 the extinction coefficient and A_1 its absorption relation for the specified spectral region, and also let y be the unknown concentration of the other colouring matter, ϵ_2 the extinction coefficient of its solutions and A_2 its absorption relation for the same spectral region, then

$$\frac{x}{\epsilon_1} = A_1 \text{ and } \frac{y}{\epsilon_2} = A_2 \text{ so that } \epsilon_1 = \frac{x}{A_1} \text{ and } \epsilon_2 = \frac{y}{A_2}.$$

Substituting these values of ϵ_1 and ϵ_2 in equation (1), then

$$\epsilon_{1\cdot 2} = \frac{x}{A_1} + \frac{y}{A_2}$$

In order to calculate x and y, we must have another relation between these two quantities. This can be determined by ascertaining the extinction coefficient of the same mixture in another spectral region. So, that if $\epsilon'_{1.2}$ is the observed extinction coefficient of this mixture and A'_1 and A'_2 the corresponding absorption relations for the new spectral region, then

$$\epsilon'_{1.2} = \frac{x}{A'_1} + \frac{y}{A'_2}$$

and from these equations x and y are obtained,

$$x = \frac{(\epsilon'_{1.2}, A'_2 - \epsilon_{1\cdot 2}, A_2) A_1, A'_1}{A_1, A'_2 - A_2, A'_1}$$

$$y = \frac{\epsilon_{1.2}A_1 - \epsilon'_{1.2}, A_2 . A'_2}{A_1, A'_2 - A_2, A'_1}$$

and x and y will be the weights of the two colouring matters in 1 c.c. of the mixture.

Theoretically, it is possible to determine three constituents in one solution by working in three different spectral regions; but G. and H. Kruss have shown that in practice the results are unreliable.

Medico-legal Examination of Blood

The appearance of blood-stains is very variable. The shape should, however, be carefully observed, so that an opinion can be formed as to whether the spot is a smear, a drop, or a splash. In the case of a fabric a knowledge of the side on which the blood fell may be of importance. Blood-stains on fabrics are generally dull, but on polished metal they have the appearance of dark shining spots, and are readily removed.

On making a preliminary inspection of any article supposed to be stained with blood, careful record should be made of the position in which suspicious marks occur, and it should be noted whether any attempt has been made to wash away the stain. In the case of articles of clothing, the positions of these marks should be indicated by inserting safety-pins. Spots on wood or metal may be surrounded with a circular mark made with a black-lead pencil, and similar means should be adopted in other cases to facilitate the subsequent operations. A convenient plan in many cases is to make a sketch of the article and mark on it with red ink the positions of the stains on the original article. Means must also be taken to allow of the certain recognition of the article on subsequent occasions, as in the witness-box, where the following questions may be asked:

1. Is the stain blood?
2. Is it human blood?
3. From what part of the body did the blood flow?
4. What is the age of the stain?

To the last two questions it is rarely possible to give a definite answer.

In the examination of a stain on any surface, material such as hair or substances other than blood should be removed and preserved. For example, it is usually not difficult to distinguish between the

hairs of man and other animals by the use of the ordinary and polarising microscope since it is easy to obtain fresh hairs for comparison (cf. Mitchell, *J. Soc. Chem. Ind.*, 1930, **49**, 451). Blood from the stomach often contains epithelial cells, and that from abscesses fat, cholesterol and pus-cells. The presence in suspected stains of spermatozoa, vaginal epithelium, fæcal or biliary matter, brain-tissue, may often have great significance.

The colour of blood-stains varies according to the age of the stain, the quantity of blood, and the nature and dye of the stained fabric. As a rule, recent stains are red, old ones brown in colour. The appearance of a stain varies with daylight or artificial light, according to the nature and colour of the material on which the blood has been effused. On metals such as iron or steel, the stain appears as dark shiny spots or smears which, when dry, are often fissured. Many substances stain like blood; for instance, jams, solutions of vegetable colouring matters, highly coloured urines, fæcal matter, rust-spots, organic iron salts, etc., and on certain fabrics even grease or paint, pitch or rubber-solutions. The whole examination of a blood-stain should be carried out systematically, and the stains carefully examined with a hand-lens before any further procedure. This would differ according to whether the stain is recent or old. If the former, the blood-pigment will be more or less soluble, and the object of the treatment should be such as to afford a solution which can be both examined under the microscope for corpuscles and by other methods for the hæmoglobin. In an old stain the corpuscles are disintegrated and the hæmoglobin is present in some form such as hæmatin. In recent stains, the piece of material is cut out and treated with some solution which has a similar osmotic pressure as blood plasma, in order that the corpuscles may retain their normal size and not be swollen or crenated. A good solution to use is Pacini's fluid—a 10% solution of chloral hydrate, or 1 part of glycerin in 7 of water, or 0.85% sodium chloride; but even for recent stains, if the garment has been ironed or the dye of the fabric has acted as a mordant, or if the material has been washed with soap and water the extraction by these solvents is very difficult or impossible. In the case of leather or woods rich in tannic acid, the hæmoglobin may have been so fixed as to become insoluble. For old stains various solvents, such as warm water, weak solutions of citric acid or ammonia may be used, or 2% hydrochloric acid.

Schaer (*Arch. Pharm.*, 1898, **236**, 571–579) advises 65 to 85% of chloral hydrate. In a very refractory stain a solution yielding the hæmatin spectrum may be obtained with strong potassium hydroxide. Before actually treating a suspected stain on a dyed fabric, it is desirable to submit a portion of the material, free from stain, to the action of water, citric acid, and ammonia, with a view of ascertaining whether colouring matter is dissolved which will interfere with or modify the blood-spectra subsequently observed. In the event of blood not being recognised in a suspected stain on such fabric, a portion of the material should be treated with blood, and the process applied to the authentic blood-stain so produced.

On exposure to air in a damp place, a blood-stain may become mouldy, with complete destruction of the colouring matter; but if kept dry, the hæmoglobin gradually changes into a complex mixture of methæmoglobin, hæmatin, and hæmatoporphyrin. If moderately recent, such a stain will be brown, and will dissolve with difficulty and probably incompletely in cold water, yielding a dirty brown solution. The alteration of blood-stains takes place far more rapidly in the atmosphere of a town, and especially in a room where gas is burnt, than in the pure air of the country. Contact with sweat may cause the hæmoglobin of a blood-stain on a dirty garment to undergo very rapid alteration into hæmatin. Such facts should be taken into consideration in forming any opinion as to the age of a blood-stain from its colour, solubility, and spectroscopic examination.

The object of all solvents is to remove material from the stain which shall give evidence of the presence of blood by one or more of the following methods:

1. Microscopical recognition of corpuscles.
2. The chemical test for blood, which depends upon certain colour-tests, *e. g.*, guaiacum or benzidine, etc. (see pp. 31, 32).
3. The production of hæmin and hæmochromogen crystals (pp. 60, 62, 105).
4. The spectroscopic recognition of the pigment.
5. The biological test for blood for specific kinds of blood (p. 110).

In order to examine a supposed blood-stain to the best advantage, it is desirable to remove as much of the surrounding material as possible. Thus if the stain be on cotton, linen, silk, wool, etc., a portion of the fabric on which the spot exists should be cut out. If on a

porous material, such as wood, brick, or stone, the stained substance should be scraped away for some depth, and reduced to fine powder. Stains on metal, especially on iron or steel, are most difficult of treatment, since the colouring matter is readily mordanted and rendered insoluble by the iron. Sometimes, on drying the article thoroughly, the incrustation will peel off, but if not, it must be removed by scraping.

On whatever material the stain may have been, when the suspected portion has been more or less separated in the above manner from the surrounding material, a portion of the coloured substance should be treated in a watch-glass with a few drops of 0.85% sodium chloride and allowed to stand a rest for a short time. If the stain is at all recent, and the colouring matter has not been mordanted in any way, the liquid will acquire a more or less distinct red or brown colour. In this case it is probable that the colouring matter of the blood is, in part at least, in a soluble form, and the subsequent examination will be simple. By preference, the liquid should be decanted from any insoluble residue. If necessary, the liquid may be filtered, but this operation should be avoided if possible. If the decanted or filtered liquid be perfectly colourless, evidence of blood may still be obtained from the insoluble portion. In such a case, as already stated, special means must be employed to effect the solution of the colouring matter. If a blood-stain has been strongly dried before a fire, or otherwise heated, so as to coagulate the albumin, the colouring matter is rendered insoluble, and cannot be extracted by treatment with water, citric acid, or cold ammonia. On heating the stain in dilute ammonia or sodium hydroxide, the hæmatin is readily dissolved, and may be detected by reducing it to hæmochromogen, either with or without previous concentration of the solution.

The same method may be employed for extracting the colouring matter from a blood-stain which has been washed, the liquid being subsequently concentrated by evaporation or neutralised and precipitated with zinc acetate.

Von Fürth (*Z. angew. Chem.*, 1911, **24**, 1625) recommends a combination of Leer's pyridine test with leuco-malachite green. The object to be tested is boiled for a few minutes with a few drops of 50% potassium hydroxide to which a few drops of alcohol have been added. After cooling, the liquid is extracted with pyridine. The pyridine layer is separated and the solution again shaken with potassium

hydroxide after which about 1 c.c. of the solution is run on to a filter paper on a glass plate. This is then treated with a solution of leuco-malachite green to which 1% hydrogen peroxide has been added.

In cases where the colouring matter remains obstinately fixed on a fabric, probably from combination with the mordant, Sorby recommended that the stain should be digested in dilute ammonia (in a watch-glass), and the liquid squeezed out repeatedly by a pair of forceps, and ultimately between the finger and thumb. The thick, turbid, unfiltered liquid is then reduced in the ordinary manner, and examined for the hæmochromogen bands, using lime-light or concentrated sunlight if necessary. By operating in this manner, Sorby succeeded in detecting blood in a stain 6 years old on brown cloth. When the tube containing the turbid liquid was kept in such a position as to allow the suspended matter to settle out, no absorption-band was produced by the supernatant fluid, the colouring matter evidently existing in the insoluble deposit. Hence, in such cases any process of deposition or filtration is inadmissible. The effect of the insolubility must be overcome by increasing the intensity of the light, and not by removing the deposit.

The spectroscopic recognition of blood on *rusty iron* is somewhat difficult. For reasons not thoroughly understood, ammonia and citric acid sometimes fail to dissolve the colouring matter. In such cases glacial acetic acid aided by heat will sometimes be found successful, but a more certain plan is to heat the spot to 50° with a cold-saturated solution of borax. The solution obtained should be treated with acetic or citric acid, filtered if necessary, reduced, rendered alkaline, and examined for the spectrum of hæmochromogen.

The recognition of blood in suspected stains on *leather* presents peculiar difficulties, since the presence of tannic acid so mordants the blood that neither water nor citric acid will dissolve the colouring matter, and ammonia produces an inconveniently dark solution. If the stain is on the surface of the leather, and has never been wetted, a thin shaving should be cut off, so as to obtain as much blood and as little leather as possible. The shaving should then be bent with the stained side outward, and placed on the mouth of a cell-tube[1] filled with water, in such a manner that the suspected blood-stain shall be in contact with the solvent without the rest of the leather becoming wetted. In this manner the dissolved colouring matter sinks to the

[1] This is made by cementing a piece of glass tubing, 1–2 cm. long and 1 cm. diameter, on to a microscope slide so as to form a capsule.

bottom of the cell without coming in contact with the rest of the leather. After removing the shaving, the liquid in the cell may be examined in the usual manner.

If a blood-stain on leather has been wetted, the foregoing method is inapplicable. In such cases Sorby recommended that the stained leather should be digested for a considerable time in water containing 2% by volume of hydrochloric acid. The liquid is then poured off (not filtered) and treated with ammonia in excess, by which it acquires a purple or neutral tint. This colour is intensified by the iron reagent which Sorby used for reduction, but it is evident that the presence of tannin renders this treatment unsuitable. Stannous chloride or ammonium sulphide can be substituted with advantage, and there will then be little difficulty in recognising the absorption-band of reduced hæmochromogen. (For methods of reduction see p. 64.)

Graham-Smith and Sanger (*J. Hygiene*, 1903, 258) found that leathers are mostly acid, and extracts of acid reaction give a precipitate with blood. After neutralisation, the precipitin test (p. 110) can be applied. If blood is dried on thick polished yellow leather, it is quite impossible to obtain the precipitin test.

Earth and soil-stained clothes render blood completely insoluble. Sorby recommended extraction with ammonia, and examination of the turbid solution with an intense light. A saturated solution of borax, as recommended by Dragendorff, may be advantageously substituted for ammonia. 1 part of blood absorbed in 200 of peat can thus be detected.

Very few colouring matters yield absorption-spectra which can be mistaken for those of blood, even by the inexperienced observer, and these can be absolutely distinguished by the application of reagents.

The petals of the red variety of *Cineraria* contain a colouring matter the spectrum of which exhibits two absorption-bands in the green, similar in position to, but differing in relative width from, those of oxyhæmoglobin. The bands of *cineraria* are completely altered on adding ammonia, while those of oxyhæmoglobin are unaltered.

Turacin, the copper-containing colouring matter present in the feathers of the turaco or plantain-eater exhibits a spectrum similar to that of blood, but it does not yield the spectrum of hæmochromogen on reduction.

A solution of *cochineal* in alum shows absorption-bands somewhat resembling those of oxyhæmoglobin, which are rendered more intense on adding ammonia. On subsequently adding excess of boric acid, the cochineal bands shift to the blue end of the spectrum, whereas those due to blood are unaffected by this treatment. The points of maximum absorption for the bands of carminate of ammonia are:

$$\lambda = 560\mu\mu$$

$$\lambda = 518\mu\mu.$$

FIG. 24.

A solution of *soluble indigo* when *hot* exhibits a spectrum not unlike that of blood, but it is decolorised by treatment with alkali and a reducing agent.

Lac-dye, *alkanet*, *madder*, and *alizarin*, when dissolved in alum, exhibit absorption-bands which distantly resemble those produced by the colouring matter of blood. Alizarin Red W, in alkaline alcohol shows three bands, and the position of maximum absorption is

$$\lambda = 610\mu\mu$$

$$\lambda = 559\mu\mu$$

$$\lambda = 518\mu\mu.$$

The spectra are all modified by treatment with ammonia, and the bands are destroyed on adding sodium sulphite, whereas the absorption-bands of blood are relatively unaffected by such treatment. *Rosaniline* (magenta, fuchsin), also, is completely decolorised by sulphites, and the colour is not restored by exposure to air.

Hæmin Test.—In 1853, Teichmann showed that the demonstration of blood crystals, known often as "Teichmann's crystals," was possible with minute traces of blood. The crystals from the blood of all animals are identical, brown rhombic prisms (Fig. 24). The test is made as follows: the dried suspected material is rubbed up on a microscope slide into a powder. To this glacial acetic acid is added, so as to make a paste, then add a few drops more acid, and cover

with a cover-glass. The quantity of acid should be sufficient to form a thin layer between the cover-glass and slide. The slide is now heated with a small Bunsen burner till it just boils. (Boiling must not be continued.) On cooling, in about 2 minutes, the dark brown crystals are seen with the microscope (magnification 1–300). If they are not present, the mixture is heated again to the boiling-point, care being taken that there is a sufficiency of acetic acid.

If the blood-stain is some years old, a minute crystal of sodium chloride or potassium chloride may be rubbed up with the paste. The addition of chloride was originally suggested by Virchow. It is not necessary for recent blood, as there is sufficient of this salt in the plasma. Too much salt interferes with the test.

According to Lewin and Rosenstein (*Virch. Arch.*, 1895, 173), the following are the conditions under which the hæmin test succeeds:

1. The blood must be dry. If liquid, it should dry in the air, not over a flame.

2. Oxyhæmoglobin, hæmoglobin, methæmoglobin, carboxyhæmoglobin, and hæmatin all yield crystals.

3. Hæmochromogen and hæmatoporphyrin yield no crystals by this method. In some meat juices the blood colouring matter is in the state of hæmochromogen.

Rose showed that when blood is mixed with iron-rust the test fails. Stains on rusty iron may, therefore, fail to give the test, or only the most superficial layers may do so. For an explanation, it is probable that in the presence of iron oxide no hydrochloric acid is formed from sodium chloride by the action of acetic acid.

$$(1)\ Fe(OH)_3 + 3C_2H_4O_2 = Fe(C_2H_3O_2)_3 + 3H_2O.$$

$$(2)\ Fe(C_2H_3O_2)_3 + 3NaCl = FeCl_3 + 3NaC_2H_3O_2.$$

The hæmin test is said to be negative for stains treated with soap and water. However, Lewin and Rosenstein find that blood-stains treated with boiling potassium hydroxide for 20–25 minutes, or washing the stain with soap and water, may yield hæmin crystals.

Beam and Frank (*Biochem. J.* 1915, **9**,) have introduced a much more reliable method of carrying out the haemin test, which should always be tried should the ordinary method fail. They contend that the failures which sometimes occur in applying Teichmann's test are due chiefly to the too rapid evaporation of the solvent, and, to

a less extent, to interference of the albuminous matter of the blood with the crystallisation.

They carry out the test as follows:—

"A small quantity of the suspected material is placed at the bottom of a flat arsenic sublimation tube, about 6 by 3 mm. and 35 mm. long. A thin round ignition tube will answer if no flat tube is procurable, but for examination under the microscope the flat form is obviously better. A few drops of acetic acid containing from 0.01 to 0.1% of sodium chloride are added, and a very fine cotton thread adjusted so that its upper end is near the top of the tube and the lower end reaches the bottom of the liquid. The thread should be everywhere in contact with the tube, to which it adheres readily by being moistened with the liquid. The adjustment is readily made by means of a glass rod with the end drawn out to a point. The tube is now placed in a rack, or supported by forcing its base into a small blob of plasticine, and allowed to remain until crystallisation has taken place. Before placing the thread in position, the solution of the blood may be aided, if desired, by cautiously heating the tube over a small flame, loss of liquid by explosive boiling being prevented by holding the finger tightly over the mouth of the tube. While such heating appears rarely to be essential, the long contact with the acid in the cold sufficing for solution in practically all cases, it is nevertheless advisable, in that it gives at once an indication of the strength of the blood solution obtained. For the best results this should be quite weak.

"The clear liquid, filtered by its passage through and along the thread, is slowly drawn up by capillary action to the mouth of the tube. Under these conditions evaporation takes place so slowly that, even when the temperature of the atmosphere is above 38°C., it is complete, even if the tube is held in an upright position, only after 12 to 24 hours or more. The rate of evaporation is readily controlled, within wide limits, by altering the dimensions of the tube and the angle at which it is supported.

"The crystals usually begin to appear on or near the upper half of the thread, and of sufficient size to be distinguished with a power of 75 diameters, in about half an hour, and they ultimately become so large that in place of requiring a magnification of 250–300 diameters, as usually recommended, they may readily be seen with one of 25 diameters or even, in many cases, with a good hand lens.

The crystals are best viewed against a dull white background. A piece of white paper is suitable. The extraordinarily large size of the crystals is due, in great part, to the slow evaporation of the liquid; but it is also a result of the removal by the thread of the interfering action of the blood albumen on crystallisation. Examination of the thread at the end of the experiment shows that the soluble, non-crystallisable constituents of the blood are carried to its upper end, and that the maximum growth of haemin crystals is found lower down. If the solution is sufficiently weak, the first crystals form at the point of greatest concentration—the upper end of the thread; but as the albuminous matter accumulates these cease to grow, and the largest and most numerous crystals are found, later, about midway down the tube. For the best results the following precautions should be observed:—The tubes should be clean and dry. The thread should be clean and as fine as can be obtained. A good plan is to take No. 80 'six cord sewing cotton' and to untwist it, using one of the cords for the test.

"The solution of the blood in acetic acid should not be too concentrated, but very weak. The liquid should only be faintly pink. This insures large crystals and clean fields. If the method is to be applied experimentally to fresh blood, the latter must be completely dried before the addition of the acid, and especial care should be taken to work with very dilute solutions." Of the reagents that have been recommended for use in Teichmann's test acetic acid is by far the best, and the only one on which complete reliance can be placed if the method of evaporation is employed. Acetic acid, without any salt, suffices for the test with blood stains—fresh or old—provided the stains *have not been extracted with water*. It is best, however, to use acid containing a trace of salt. No advantage results from the substitution of other halide salts for sodium chloride."

Many observers consider that the formation of hæmochromogen crystals by the pyridine method given on page 62 and their examination with the micro-spectroscope affords a delicate and certain test for blood-colouring matter. The ruby-red colour of the crystals under the microscope is very characteristic, and a minute amount of blood suffices for their demonstration. It is difficult to obtain the crystals as sharply defined as is the case for hæmin.

The test is carried out as follows: (Donogany, *Maly's Jahresber. f. Tierchem.*, 1894, **23**, 126, and *Virch. Archiv.*, 1897, **148**, 234). Some

drops of defibrinated blood are mixed on a slide with a few drops of pyridine, and then some drops of ammonium sulphide. The mixture is covered with a cover-glass. The blood of all animals yields crystals. Carbon-monoxide blood yields crystals of carboxy-hæmochromogen. The crystals of hæmochromogen are red bundles or masses of needles. In their preparation no air-bubbles should be allowed to appear., The test is of diagnostic value for blood or hæmoglobin.

The examination of blood in order to throw light on the cause of death by asphyxia, by carbon-monoxide poisoning, by cyanides, sulphuretted and arseniuretted hydrogen, by potassium pyrogallate and chlorates, may be required.

In asphyxia, most of the blood-colouring matter, both in the arteries and veins, is in the state of reduced hæmoglobin. In poisoning with carbon monoxide, the blood contains a mixture of oxyhæmoglobin, hæmoglobin and carboxyhæmoglobin.

In death from hydrogen sulphide the compound with hæmoglobin is of an evanescent character, but may show the spectrum described on page 50.

In prussic-acid poisoning, the blood is often scarlet in colour, and the spectroscopic features of the pigment are described on page 48.

In poisoning with fluorides or hydrogen fluoride, the spectrum may exhibit the features described on page 55.

In poisoning with chlorates, pyrogallic acid or arseniuretted hydrogen, the blood may contain a variable amount of methæmoglobin.

To differentiate the blood of man from that of lower animals by examination of the appearances and properties of the corpuscles is a matter of practical impossibility, except in very favourable cases where the blood corpuscles can be examined and measured (see p. 5).

Mammalian blood is non-nucleated, in that of birds, fishes, reptiles and amphibia, oval nucleated corpuscles are present. These facts are of slight medico-legal importance; it is, however, possible to state whether blood is that of a mammal or not.

Various observers have attempted to differentiate bloods of animals by the properties of the haemoglobin, which is no doubt specific. Reichert and Brown have published careful and extensive

studies of the crystalline form of the haemoglobin from a great number of animals.

(Reichert and Brown: the differentiation and specificity of corresponding proteins, etc., the crystallography of haemoglobin, No. 116. *Carnegie Institution publication*, Washington, D. C.)

And Landsteiner and Heidelberger have determined the solubilities of different haemoglobins (see p. 17); but unless fresh blood is available, the methods are not of great importance from a medico-legal point of view.

Another test which is also only of confirmatory value is that of Beccadelli (*Arch. Farm. Sper.*, 1923, **36**, 137; *Chem. Abst.*, 1924, **18**, 406) who has shown that the presence of blood has a pronounced influence upon the reduction of silver nitrate by formaldehyde, and that the colour of the colloidal silver varies with the species of animal. For the identification of the blood in stains on fabrics a fragment (about 5 sq. mm.) is cut out and macerated for 24 hours in water at the ordinary temperature. Then about 0.5 c.c. of the extract is treated with 0.5 c.c. of formalin (38–40%), 0.5 c.c. of 0.75% silver nitrate solution, and 0.5 c.c. of ammonia (sp. gr. 0.925), and the tube is inverted several times and allowed to stand. Human blood gives an amber-yellow coloration in 16 hours if the stain is not recent, a turbidy if recent. Failure to obtain this colour may indicate the presence of the blood of lower animals. Thus ox blood produces a brass-yellow coloration and dogs' blood a brick red. The differences are attributed to differences in the size of the colloidal particles of silver.

The researches of Uhlenhuth, Schütze, Wassermann, Nuttall and others have shown however that the blood of man can be distinguished from that of other animals, and these from one another.

The Precipitin Test for Blood.—The serum of blood must be regarded as resulting from the death of blood; it has the same relation to plasma that killed has to living protoplasm. The serum obtained from the blood of normal animals and that from animals which have been subjected to various procedures, such as the injection of the blood of other animals, foreign proteins, or solutions of ferments, yield a normal or modified serum respectively. In 1899 Tschistovitch, working with eel's serum, showed that rabbits which were injected with this possessed a blood the serum of which produced a

remarkable cloudiness on admixture with eel's serum. In other words, an injection of eel's serum produced in the rabbit's serum a substance which, reacting outside the body in a test-tube with eel's serum, caused a precipitate or cloudiness. This observation was confirmatory of work by Bordet, and the reaction is often spoken of as that of Bordet-Tschistovitch. This is the basis of the precipitin or biological test for blood (Uhlenhuth, Ziemke) and, as used first by Nuttall, is one which has been employed with great success for determining the relationships which exist between different animals. (Nuttall, "*Blood Immunity and Relationship*," 1904; Cambridge.) When blood is injected into an animal—the rabbit is generally chosen for this purpose—the substances developed in its blood serum are known as precipitins. These are not known by any definite chemical or physical tests, but they are specific. If dog's blood be injected, then the rabbit serum contains a precipitin specific for dog's blood. If fowl's blood, then a precipitin specific for the fowl; human blood, a precipitin specific for man. In all these cases a cloudiness or precipitum results only when the actual blood-containing precipitin is mixed with the blood of the animal which originally supplied the blood for injection. The various sera are termed anti-dog, anti-fowl or anti-human for the above examples, but it is the case that a number of other anti-sera may be obtained by a similar method of experiment. An example may make this clear.

Anti-human serum was obtained by injecting into the peritoneal cavity of rabbits, every 6 or 8 days, 10 c.c. of defibrinated human blood. At the end of a month the rabbit was killed, its blood collected, and allowed to clot. The serum contained a specific precipitin as the following results show:

On using 3 c.c. of blood, diluted 1:20 or 1:40, and adding 1–2 drops of the anti-human serum, a white cloud slowly forms and increases, finally settling at the bottom of the small test-tube (capacity about 6 c.c.). The reaction is accelerated by a temperature of 37° C., but apparently in no other way. (Graham-Smith and Sanger. *J. Hygiene*, 1903.)

Diluted Blood.	
1. Human (fresh)	Precipitum (positive).
2. Human (dried)	Precipitum (positive).
3. Frog	Negative.

4. Fowl	Negative.
5. Guinea-pig	Negative.
6. Cat	Negative.
7. Human Serum	Positive.
8. Horse	Negative.

In Uhlenhuth's original list (*Deutsch. med. Wochensch.*, Feb., 1901; Sept. 1902) 19 different bloods were tested with anti-human serum. A positive reaction (formation of precipitum) occurred with human blood, but not with that of the dog, cat, pig, sheep, deer, fallow deer, horse, donkey, hare, rabbit, guinea-pig, rat, mouse and four different birds. Wassermann and Schütze (*Berl. klin. Wochensch.*, Feb., 1901) give details of 23 observations; only the blood of man and baboon gave positive results with anti-human serum. (See also Ziemke, *Deutsche med. Wochensch.*, Jan. and Oct., 1901.)

Preparation of Antisera.—A rabbit is placed on its back and the abdomen lathered over with liquid soap containing lysol. A small area on the left side at the lower end of the abdominal region is then shaved. This area is disinfected with acetone. The skin and muscle are gathered together with the disinfected fingers, care being taken that the gut is not included, and the sterile needle of a hypodermic syringe holding about 10 c.c. of sterile blood, or serum, or defibrinated blood, is thrust through the skin and muscle into the peritoneal cavity, care being taken to keep the needle horizontal and to make sure that this enters the abdominal cavity and does not wound the gut. After injection the needle is withdrawn, the skin dried, and the wound treated with some disinfectant dressing such as solid boracic acid powder. 5 or 6 injections are made at intervals of 2–4 days, and the animal killed 7–12 days after the last injection. The animal is chloroformed, the thoracic cavity opened under aseptic precautions, the heart is incised and the blood allowed to flow into the cavity whence it is removed with sterile pipettes into a sterile dish. The blood is allowed to clot and the serum is pipetted up into sterile test-tubes and then transferred to sealed bulbs of small size. Any serum which separates on further shrinking of the clot should be passed through a Chamberland filter to prevent bacterial development. Opalescent sera are of little use in carrying out the precipitin test.

Medico-legal Test for Blood.—The precipitin test was first employed by Uhlenhuth. Having prepared anti-serum by the

repeated injections of human blood into rabbits, and diluted the suspected human blood with an equal volume of 1.6% sodium chloride he added 6 to 8 drops of anti-human serum. The reaction is carried out in small test-tubes holding about 5 c.c. After mixing the liquids the tubes are placed in the incubator at 37°. For old dried blood-stains he first applies the guaiacum and hæmin tests, and then, by using various anti-sera which contain the specific precipitins for the blood of different animals, the kind of blood is identified.

According to Nuttall, who used dilutions of human blood 1:100 of normal saline 0.85% and equal volumes of 1:100 diluted antihuman serum, the positive reaction is obtained with old blood (2 months), putrified blood, or blood mixtures of human and other bloods. The test is extraordinarily delicate, dilutions of human blood 1:16,000 reacting positively.

Different observers vary somewhat in the details of the procedure they use in applying the test.

Sir W. H. Willcox describes his method as follows:—

"In performing the test on blood stains, the suspected stain is treated with a solution of salt of the same strength as that of blood serum (normal saline). The solution of the stain is clarified by centrifugalisation, and to the clear solution in different small test tubes a few drops of different anti-sera are respectively added. If the stain is one of human blood, a precipitate will be given with a human antiserum, but not with a sheep or ox or other anti-serum than man. This test is a reliable one provided that a series of anti-sera are available, and provided also that the blood stain is sufficiently large to give a solution of blood serum of sufficient strength to give a *marked obvious precipitate* when the test is applied." (*Circumstantial Evidence*—Wills, 1912, p. 165.)

Lt.-Col. D. W. Sutherland of the Imperial Biological Institute, Calcutta, describes this method thus:—

"When the observer desires to determine the origin of a blood stain he makes an extract of it by soaking the stained fabric or scrapings of the stain in physiological salt solution. Some stains are hard to extract, and for these the addition of a few drops of solution of potassium cyanide to the salt solution in which they are immersed is a good plan. The stain extract is then tested as to its alkalinity and acidity. If it be acid it must be rendered neutral or slightly alkaline by the addition of a drop or two of a weak solutions of

caustic soda or potassium cyanide. If it be strongly alkaline, as it will be if the cyanide solution has been added to hasten extraction, it must be rendered only slightly alkaline by the addition of a drop or two of a solution of tartaric acid. The extract having been thus treated is diluted with salt solution until it corresponds to a 1:1,000 dilution of serum. The observer, having made water clear extracts of all the blood stains that have to be examined, sets these out in taper tubes, and to the contents of each adds two drops of an anti-human serum, which he knows is on the day in question specific and highly potent. Those extracts which within 20 *minutes* give a distinct reaction are noted, and the stains from which they have been obtained are proved to have been caused by human blood." (Lyon: *Medical Jurisprudence for India.* L. A. Waddell, Calcutta, 1914. Article, Blood stains. Lt.-Col. D. W. Sutherland.)

It is essential that the solution to be tested should be free from acid or acid salts, otherwise a precipitate would probably form with *any* anti-serum.* Probably the most satisfactory method of extracting any stain of acid character is by means of saline containing in suspension a trace of some insoluble base such as magnesia. Extracts should be made only with saline, not with tap water or distilled water, as these are liable to render antisera turbid. On the other hand, excessive alkalinity would be liable to retard or even prevent the formation of a precipitate, and antisera from different rabbits should never be mixed.

The concentration of the blood extracts to be tested should also not be very far from the maximum dilution of the blood serum in normal saline with which the anti-serum to be used will produce a definite turbidity within a specified time. The concentration of the blood extracts may be roughly tested by comparing the turbidity produced by the addition of a few drops of dilute nitric acid to a small quantity of the extract, and to solutions of blood sera of lesser strength.

The reason for this is that precipitin anti-sera prepared as described contain anti-sera for many proteins, and with powerful anti-sera and strong blood solution a slight turbidity due to a precipitin common to all mammals might be observed.

Authorities appear to differ as to time in which turbidity should form. Sutherland observes in 20 minutes, other workers adopt a shorter limit.

In order to overcome the difficulty of preparing clear extracts of very small stains without loss or over-dilution of the extract, W. M. Colles of the Ministry of Justice, Cairo, has devised a micro-precipitin test. A full account of the procedure, which is stated to have given satisfactory results after some years' trial in Egypt, will be found in Lucas's *Forensic Chemistry*, Edward Arnold, London, 1921, pp. 35–39.

Roche Lynch (*Analyst.*, 1928, **53**, 9) gives the following directions for applying the test, and recommends the "layering" method as the most satisfactory, since by layering the unknown extract above the antiserum, so that a sharp ring is formed at the junction of the liquids, the faintest trace of precipitin formation can be observed when the tube is examined in an oblique shaded light against a dark background.

"The antiserum is used undiluted, but the antigen or unknown is diluted, so that the concentration is not greater than 1 in 1,000. A concentration of 1 in 1,000 will completely avoid any negative reactions due to zone reactions, and will avoid false positive results from group reactions except in the case of very closely related species. Generally one puts up a series of dilutions of the unknown extract from 1:1,000 to 1:10,000, so that one can observe the gradual fading of the reaction.

"The antisera and extract must be absolutely clear, which is ensured by filtration or centrifuging, or both. The complete test, no matter which technique is used, requires the following tubes for control:—

(1) Antiserum and saline used for dilutions.

(2) Antiserum and portion of article under examination free from blood.

(3) Antiserum and known human blood (1:1,000).

(4) Antiserum and animal blood, say, horse (1:100).

(5) Antiserum and animal blood, say, sheep (1:100).

(6) Antiserum and animal blood, say, dog (1:100) (or any animal likely to be put forward by defence).

(7) Normal rabbit's serum and extract of stain under test.

(8) Antiserum and borax or cyanide solution if such was used for rendering the stain soluble.

(9) Antiserum and Unknown extract estimated dilution (1:1,000).

(10) Antiserum and Unknown extract estimated dilution (1:5,000).

"The tubes are allowed to stand at the laboratory temperature for 20 minutes, and are then read. With good antisera, tubes 3, 9, and 10 will have given positive results if the stain is human. The other tubes (Nos. 1, 2, 4–8) should be completely negative, but at the end of an hour or more it is possible that tubes 4 to 6 may show some slight traces of precipitin formation.

"If borax or potassium cyanide has been used for extracting the stain it is usual to neutralise the extract with tartaric acid, and these chemicals must appear in the list of controls."

The results of the precipitin test are accepted in England and most of the Dominions and have been recognised in France and Germany for some years. Nevertheless, except in the hands of those having constant practice in the procedure, or engaged in seriological investigation, the test presents many pitfalls. Thus, although the method outlined above gives satisfactory results in most cases, yet from time to time cases occur in which a great refinement of technique is required for a decision. For further information as to this test the following authorities might be consulted, in addition to those mentioned in the text.

Taylor: *Principles of Medical Jurisprudence.* F. J. Smith, London, 1920.

Lyon's Medical Jurisprudence of India, L. A. Waddell, Calcutta, 1914, article Blood Stains by Lt.-Col. W. D. Sutherland.

Forensic Chemistry. A. Lucas, Arnold & Co., London, 1921. (2nd Ed. 1931.)

Legal Medicine and Toxicology, Peterson, Haines and Webster, Vol. II, p. 898 et seq.

The Precipitin test for Blood, Ludvig Heletven, *J. A. M. A.*, 1918, 70, 1373.

Blood Groups.—The most recent forensic development connected with blood is the classification of bloods into different groups which can be distinguished from one another. The principle upon which the method rests is that each type of blood contains an active principle capable of reacting with other types of blood in such a way as to cause the blood corpuscles to agglutinate or form clumps. Systematic tests made by L. and H. Hirschfeld (*Lancet,* 1919, ii, 675) show that the four groups into which all human blood can be classified vary with different races. For instance, the following results were obtained:

Cases	Groups per cent			
	I	II	III	IV
Europeans, average of 4,000	4.70	41.23	12.14	41.93
Asiatics, average of 2,500	6.74	21.84	32.74	38.68

The method of ascertaining to which group a particular person's blood belongs is described by Roche Lynch and Martley (*Lancet*, 1924, **206**, 913) as follows:—"A drop of Group II serum and a drop of Group III serum are placed at either end of a microscope slide, and a little of the blood under examination is taken up on a platinum needle and mixed with each. Agglutination can be readily seen with the naked eye in a few minutes. When it occurs in both drops the blood is of No. I Group; when it is in neither it is of No. IV Group; and when it is in only one it belongs to the group of the serum in which there is no agglutination."

Martley (*Analyst*, 1928, **53**, 14) gives the following illustrative diagram to show the interactions of the cells and sera of the four groups; the crosses indicate clumping, and the dashes its absence.

	Corpuscles			
	1	2	3	4
Serum I	−	−	−	−
Serum II	+	−	+	−
Serum III	+	+	−	−
Serum IV	+	+	+	−

He points out that a person whose blood belongs to Group I is sometimes known as a universal recipient, since his serum does not cause clumping of any kind of corpuscles, whilst one whose blood belongs to Group IV is termed a universal donor as his corpuscles cannot be agglutinated by any serum. Martley's method of applying the test to blood stains consists in recovering the active principles (agglutinogens) from the stain, and using them to exhaust or diminish the agglutinising power of known serum:—

"I first take a known test serum and make dilutions of ½, ¼, ⅛, and so on, to 1 in 500 or 1 in 1,000; I then place equal volumes of these dilutions on a slide and add to each an equal volume of 1% agglutinable washed corpuscles, and note down to what dilution the clumping will be manifest. I next repeat the process with the same serum incubated with a piece of the same material, free from stain, as that on which the stain has been found; the clumping may not now extend so far down the dilutions—perhaps two or three places less than in the previous experiment. Afterwards I proceed to incubate the same serum with the actual blood stain, and if the

stain came from a group which the test serum would not agglutinate, the serum will now either cease to clump the test corpuscles, or else its power will be extinguished several places sooner than in the previous experiment.

"If definite results are obtained in the two sets of experiments, I should feel justified in identifying the blood-group of the stain; if in one set I got definite positive results, and in the other corroborative negative results, I should still feel confident in my diagnosis, but I should never be satisfied to give an opinion on purely negative evidence."

Applications of the Test.—There are two directions in which the test is of potential value. It may afford confirmation, or the reverse, of a statement made by a person accused of a crime that stains found upon his clothes are not those of the blood of a victim, but were produced by his own blood.

The other possibility is the use of the blood grouping to establish or disprove paternity. The question has been discussed at length by Martley *(loc. cit.)*, who points out that the blood-grouping of the offspring of a given couple depends directly upon the blood-groups of the parents. A parent may transmit his or her agglutinogen or the corresponding agglutinin; and can also transmit his or her agglutinin, but not the corresponding agglutinogen. Application of this rule shows that the offspring of any given pair may thus be calculated, and Martley, who gives the following table, asserts that it is not possible for two Group IV parents to have a Group II child, and that such an occurrence could "only be explained by the intervention of a third party."

Mating	Possible children	Impossible children
I + I, II, III or IV	I, II, III or IV	None
II + III		
II + II or IV	II or IV	I or III
III + III or IV	III or IV	I or II
IV + IV	IV	I, II or III

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See the lists of works under the different Sections on pp. 11, 13, 22, 31, 57, 63, 116. Also *Individuality of the Blood.* By L. Lattes. Oxford University Press, 1932.

ALBUMINOIDS OR SCLEROPROTEINS

BY JEROME ALEXANDER

According to the classification adopted by the American Physiological Society and the American Society of Biological Chemists, the term *albuminoid* should be restricted to simple proteins[1] exhibiting pronounced insolubility in all neutral solvents. Since this group includes elastin (from tendon), collagen (from bone and hide), and keratin (from hoof and horn), substances forming the chief organic constituents of the skeleton of animals, as well as of the skin and its appendages, the Chemical and Physiological Societies of England prefer the term *scleroprotein* to albuminoid.

The term *albuminoid* (literally albumin-like) has in the past been used as synonomous with "proteid," and thus included not only albumin and its congeners, but also gelatin and allied substances. Later it was proposed to disregard the term "albuminoid," and to apply the term "proteoids" to "proteids" of the gelatin group, which includes ossein, keratin, fibroin, chitin, etc.

That a classification of albuminoids on the basis of their chemical constitution is extremely difficult, if not impossible, is evident from the table on page 121[2] which shows the products of hydrolysis of some of the more important members of this heterogeneous group. Furthermore, it is practically impossible to obtain a chemically pure protein, for in colloidal substances of this kind the formation of physico-chemical, adsorption, or colloid compounds has prevented their complete separation from foreign substances, from associated proteins, and from each other. Thus "chondrigen," which has been considered to be an albuminoid, is in reality a glycoprotein (see page 190); and "chondrin," the product of its hydrolysis, also belongs to the same group. Chondrigen is apparently a combination of some sort between ossein and mucin, probably an adsorption derivative.

[1] Simple proteins are those which yield on hydrolysis only α-amino-acids or their derivatives.
[2] Taken mainly from "The Chemical Constitution of the Proteins" by R. H. Aders Plimmer, Part I, page 24.

Chemists and physicists must squarely face the fact of nature, that there are combinations of molecules less definite, less powerful, and less permanent than the true chemical combinations between atoms and between some molecules. Indeed, as molecules become larger their residual or unsatisfied electronic fields become more complicated, and as P. V. Wells puts it (*J. Washington Academy of Sciences*, 1919, Vol. 9, No. 13), "molecular systems have lost so much of their discreteness that combinations of molecules do not follow the laws of definite and multiple proportions. In such phenomena as molecular association and surface structure, the discreteness of atomic constitution begins to give place to statistical continuity." Wells classifies forces thus: *Electronic forces*, which maintain negative or valence electrons and the positive nucleus [also protons and electrons within the nucleus, J. A.] in equilibrium as a single system; *atomic forces*, which maintain two or more atoms in equilibrium as a single system; *molecular forces*, which maintain two or more molecules in equilibrium as a single system; and *molar forces*, which maintain two or more masses in equilibrium as a single system. The forces binding together protein components belong to the molecular group.

True chemical combination is not necessary to make separate units behave differently. Neither an arrow-head, an arrow shaft nor a goose feather is an arrow, though the three when put together in certain ways will make an arrow and function as an arrow. In the proteins, as with other complicated molecular groupings, we perceive the effects of *structure*, even if we do not yet understand the exact nature of the structural arrangements, which may be modified by relatively mild treatment. While the *tendency* of atoms and molecules is to reach their positions of minima of potential (which is usually a crystal space-lattice), it often happens that these tendencies are unable to effectuate themselves within measurable time. As Thomas Graham said, colloids possess *Energia*, and undergo *ageing* or *syneresis*. They have a span of life; but it may be a long one. At each stage of the process of adjustment, the "particle" presents to its milieu a specific mosaic of residual unsatisfied valencies or "free fields of force," which, for the time, dominate its behaviour. The experiments of Devaux show that if a droplet of fatty acid solidifies while floating on cooling water, the under side

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	Gelatin (Dakin)	Gelatin (Fischer, Levene and Aders; Fischer, Hart, Kossel and Kutscher)		Gelatin (Skraup and von Biehler)	Silk-gelatin (Fischer and Skita; Fischer)	Silk-fibroin (Fischer and Skita; Fischer)	Spider-silk-fibroin (Fischer)	Elastin (Abderhalden and Schittenheim; Schwarz; Kossel and Kutscher)	Spongin (Abderhalden and Strauss; Kossel and Kutscher)	Koilin (Knaffl-Lenz)	Egg-membrane of Scyllium Stellare (Pregl)
Glycine	25.5	......	16.5	12.4	0.2	36.0	35.2	25.8	13.9		2.6
Alanine	8.7	......	0.8	0.6	5.0	21.0	23.4	6.6	......	...	3.2
Valine	0.0	......	1.0	......	......	0	......	1.0	......	...	......
Leucine	7.1	......	2.1	9.2	......	1.5	1.8	21.4	7.5	...	5.8
Isoleucine	0.0	......	......	......	......	......	......	......	......	...	......
Phenylalanine	1.4	......	0.4	1.0	......	1.5	......	3.9	......	...	3.3
Tyrosine	0.01	......	0	......	5.0	10.5	8.2	0.4	0		10.6
Serine	0.4	......	0.4	......	6.6	1.6	......	......	......	...	......
Cystine	......	......	......	......	......	......	......	......	......	...	......
Proline	9.5	......	5.2	10.4	......	+	3.7	1.7	6.3	...	4.4
Oxyproline	14.1	......	3.0	3.0	......	......	......	......	......	...	......
Aspartic acid	3.4	......	0.6	1.2	......	+	......	+	4.7	...	2.3
Glutamic acid	5.8	......	0.9	16.8	......	0	11.7	0.8	18.1	...	7.2
Tryptophane	......	......	0	......	......	......	......	......	......	...	......
Arginine	8.2	9.3	7.6	9.3	+	1.0	}	0.3	......	3.6	3.2
Lysine	5.9	5	2.8	6.0	......	+	} 5.24	......	......	1.7	3.7
Histidine	0.9	......	0.4	0.4	4.0	+	}	......	......	0.1	1.7
Diaminotrihydroxydodecanic acid	......	......	......	......	......	......	......	......	......	...	......
Ammonia	0.4	......	0.4	......	......	......	1.2	......	......	...	......
Total	91.31	......	41.1	70.3	20.8	73.1	90.44	61.9	50.5	5.4	48.0

ALBUMINOIDS OR SCLEROPROTEINS.—(*Continued*)

	Keratin from ox horn (Fischer and Dorpinghaus; Mörner)	Keratin from sheep's horn (Abderhalden and Voitinovici)	Keratin, from sheep's wool (Abderhalden and Voitinovici)	Keratin, from horse hair (Abderhalden and Wells)	Keratin, from goose feathers (Abderhalden and Le Count)	Keratin, from egg-membrane (Abderhalden and Ebstein; Mörner)	Keratin, from egg-membrane of Testudo Graeca (Abderhalden and Strauss)	Ichthylepidin, from fish scales (Abderhalden and Voitinovici)
Glycine	0.4	0.5	0.6	4.7	2.6	3.9	+	5.7
Alanine	1.2	1.6	4.4	1.5	1.8	3.5	+ ?	3.1
Valine	5.7	4.5	2.8	0.9	0.5	1.1		
Leucine	18.3	15.3	11.5	7.1	8.0	7.4		15.1
Isoleucine								
Phenylalanine	3.0	1.9		0	0		+ ?	
Tyrosine	4.6	3.6	2.9	3.2	3.6			1.0
Serine	0.7	1.1	0.1	0.6	0.4			
Cystine	6.8	7.5	7.3	above 10.0		7.6		
Proline	3.6	3.7	4.4	3.4	3.5	4.0	11.8 ?	6.7
Oxyproline								
Aspartic acid	2.5	2.5	2.3	0.3	1.1	1.1	1.8 ?	1.2
Glutamic acid	3.0	17.2	12.9	3.7	2.3	8.1	3.0 ?	9.2
Tryptophane								
Arginine	2.3	2.7						
Lysine		0.2						
Histidine								
Diaminotrihydroxydodecanic acid								
Total	52.1	62.3	49.2	35.4	23.8	36.7	16 6 ?	42.0

of the droplet which has "set" in contact with water acts quite differently from the upper side which formed in contact with air.

While the albuminoids are all amorphous and insoluble in cold water, some members of the class are dissolved by boiling with water (with or without pressure), to form solutions which gelatinise on cooling. These substances may be conveniently grouped together as *collagens* or *jelly-forming albuminoids* (collagenes or gelatoids). A distinction between proteins in general and collagens which has been very generally insisted on, is that when compounds of the former class are split up by the action of somewhat dilute mineral acids, tyrosine, tryptophane, and other compounds of the aromatic series are prominent among the products of their decomposition; whereas gelatin and its congeners yield no appreciable quantities of aromatic products on similar treatment.[1] This distinction does not hold good for substances of the keratin and fibroin classes, tyrosine being present to a sensible extent in the products of their decomposition.

In the absence of a better classification, the albuminoids may be tentatively grouped as follows:

A. *Collagens* or *Jelly-forming Albuminoids:* *Collagen* and *gelatin;* from bones, skin etc. Page 128. *Chondrigen* and *Chondrin;*[2] from permanent cartilages. Page 190 *et seq.* *Isinglass;* from swimming bladders of fishes. Page 180. *Sericin* (silk-gum) from silk. Page 200.	Dissolved more or less readily by boiling water. The solutions gelatinise on cooling. Contain little or no sulphur.
B. *Fibroids:* *Elastin;* from elastic ligaments. Page 197. *Fibroin;* from silk and spiders' webs. Page 204.	Not acted on by boiling water or very dilute boiling alkali. Dissolved by stronger alkali. Unaffected by dilute acids. Contain no sulphur.
C. *Chitinoids:* *Chitin;* from external coatings of invertebra.* Page 252. *Chonchiolin;* from shells of mollusca. Page 252. *Spongin;* from sponges. Page 254.	Not acted on by boiling water or alkalis. Contain no sulphur.

[1] For this reason gelatin has been found valuable as a partial substitute for other proteins in cases of intestinal indigestion, especially in infants. See "On Infantilism from Chronic Intestinal Infection," by Prof. Christian A. Herter.

[2] Chondrigen and chondrin properly belong with the glycoproteins, but are considered here because they occur in glue and the materials from which it is made.

* F. L. Campbell (*Ann. Entomological Soc. of America*, 1929, **22**, 401) points out that chitin is "a colorless, nitrogenous *polysaccharide*. It is also found in fungi, H. Braconnot (*Ann. Chim.*, *1811*, **79**, *265–304*) having first isolated it from mushrooms." It should be recalled that recently M. Heidelberger isolated specific polysacchrides which form capsules around pneumococci.

D. *Keratins:* *Keratin;* from hoofs, horns, feathers, hair, wool, etc. Page 255. *Neurokeratin;* from brains. Page 255.	Not acted on by boiling water. Dissolved by boiling with dilute alkali hydroxide. Contain sulphur.

Collagens or Jelly-forming Albuminoids

The substances of this group, though insoluble in cold water, are by heating with water converted into soluble products whose solutions gelatinise upon cooling, providing (1) the solution is sufficiently concentrated, (2) the cooling is sufficiently profound and prolonged, and (3) providing the heating is not continued until the power of gelatinisation is lost by consequence of further degenerative changes. *Boiling* is not essential; the first "run" or extraction of glue or gelatin from pig skin, can be taken at about 49°; from calf skin, at 71°; treatment of the stock and its *p*H are factors. Thus collagen or ossein yields gelatin, "chondrigen" yields "chondrin," and sericin, the collagen of silk ("silk-glue") yields, when heated with water, a solution which, if sufficiently concentrated, sets to a jelly on cooling.

Collagen. Ossein

This albuminoid may be conveniently prepared from white fibrous connective tissue or tendons, of which it forms the chief solid constituent. The *tendo Achillis* of the ox which, according to Buerger and Gies,[1] contains about one-third of its weight of collagen, serves very well. After freeing the tendon from its sheath, it is cut into small pieces and thoroughly washed with frequent changes of cold water to remove soluble protein and inorganic salts as far as possible. It is then digested for several days in cold lime water to dissolve the mucoid cementing-substance between the fibres. The insoluble residue is washed with water, dilute acetic or hydrochloric acid, and lastly with water again. The collagen thus obtained contains small quantities of elastin.

A better product may be obtained by digesting carefully cleansed tendons with trypsin, which dissolves all the tissue elements except the true collagenous fibrils.

[1] These authors give the following composition of the fresh tissue:

Water	62.87 %	Inorganic matter	0.47	Fatty substance (ether soluble)	1.04
Solids	37.13 %	Organic matter	36.66	Coagulable protein	0.22
				Tendomucoid	1.28
				Elastin	1.63
				Collagen	31.59
				Extractives, etc	0.90

Bones also yield a collagen which enters largely into commerce under the name of *ossein*. It may be obtained by digesting bones (preferably crushed or comminuted) in cold dilute hydrochloric acid, which leaches out the calcium phosphate, etc., without affecting the ossein. After soaking in cold water to remove residual acid and calcium salts, the ossein remains as a swollen elastic mass which retains the shape of the original bone fragment.

According to Hoppe-Seyler collagen is a substance characteristic of mature vertebrates, being found in all of them except the borderline *Amphioxus lanceolatus*. Invertebrates do not show jelly-forming tissue, though some is found in the cephalopods *Octopus* and *Sepiola*. Bone formation involves the deposition of salts, mainly calcium phosphate and carbonate, in a collagenous matrix which functions as a protective colloid and inhibits crystallisation. Dr. William Miller Ord of St. Thomas' Hospital, in a remarkable but overlooked book "On the Influence of Colloids on Crystalline Form and Cohesion" (London, 1879), finds "reason to suggest that the relative compactness of bone in different classes of vertebrates is dependent upon three factors—(a), the nature of the colloid matrix; (b), the nature of the earthy salts; (c), the temperature of the body—the greatest compaction being obtained with albuminoid matrix, with a mixture of carbonate and phosphate of calcium, and with the highest temperature, as in birds." In pathological cases bone already formed may be destroyed (as in osteomalacia); or bone formation may be inhibited (as in rickets, the "English disease"). Feeding and other experiments have demonstrated that absence from the food of a substance or substances called the *anti-rachitic vitamin*, results in rickets; and further, that this "vitamin" must be exposed to ultra-violet radiation (either by raying the food before feeding or the animal after feeding) to enable it to produce its curious effect in determining the deposition of bone salts. Unbelievably minute quantities of the vitamin suffice, and cod-liver oil is especially rich in it. The efficacy of the "old-fashioned" treatment for children of cod-liver oil and sunlight is thus scientifically justified, and the danger of a murky smoked-dimmed atmosphere which obstructs ultra-violet radiation, becomes manifest. Present research indicates that the precusor of the anti-rachitic vitamin is *ergosterol;* and its changes on insolation and function in bone formation seem to be rather physico-chemical than chemical. Thus Pauli and

Samec found that serum albumin increases the solubility of $CaCO_3$ 475% and Ca phosphate 90%; but with albumoses (cleavage products of albumin) the figures are reversed.[1]

According to J. J. Andeer (*Compt. rend.*, 1895, **126**, 1295), an aqueous solution of phloroglucinol acts as a powerful decalcifying agent on the bones of animals, but is without action on the most delicate organic tissue. If, in addition to this, the bones are treated with hydrochloric acid, the residual ossein will contain no trace of calcium phosphate or carbonate.

Collagen must not be considered as a definite chemical substance, for its composition varies with its origin.[2] It is insoluble in water, saline solutions and dilute alkalies. In dilute acids or alkalies it swells up to a gelatinous mass without undergoing solution, and on exact neutralisation of the acid returns substantially to its original condition. Thus in preparing collagen from bone for the glue boiling operation, it is preferably degreased, crushed to ¾″–1″ mesh, and then swollen or plumped in dilute soda or acid (H_2SO_3, HCl), or in lime water.

Collagen, like gelatin, fibrin, and many other proteins, is extremely sensitive to very slight changes in hydrogen ion concentration or effective acidity (pH), and shows a minimum swelling capacity at what is believed to be its isoelectric point.

The marked insolubility of collagen in all ordinary protein solvents is analogous to the behaviour of cellulose among vegetable substances; collagen and closely allied substances are the principal constituents of the rigid frame of animals, whilst cellulose and its allied substances form the chief structural tissues of plants.

Upon heating with water, collagen or ossein slowly dissolves with conversion into gelatin, the solution of which, under proper conditions, gelatinises on cooling. The conversion is facilitated by pressure, dilute acids, and proper soaking, swelling, or "plumping" the collagen, especially if it has previously been dried. It is a mistake to speak of this process as "boiling," for if full boiling temperature is reached, the gelatin first formed undergoes further change into *gelatin-peptones* or *gelatones*, the solutions of which do not

[1] See also H. Bechhold, "Colloids in Biology & Medicine," R. E. Liesegang," Beitrag zur einer Kolloidchem. Theorie des Lebens"; and "Colloid Chemistry, Theoretical and Applied," J. Alexander, Vol. II (Biology & Medicine).

[2] *Abderhalden* states regarding collagen (page 149, Ed. 1906) "Es ist nach mehreren Beobachtungen keine einheitliche Substanz und dürfte bei verschiedenen Tierarten und in verschiedenen Organen ein verschiedenes sein."

gelatinise on cooling. It is difficult, however, to remove all gelatinising tendency, for even "cook out" liquors, set to a weak gel. This change also occurs at lower temperatures, but more slowly, so that in converting collagen into gelatin the operation must not be unduly protracted by too low a temperature. The most desirable temperature is about 55° C. for the first extraction, but this varies with the condition of the collagen, *p*H being an important factor. The final extraction is heated to boiling.

The change of collagen successively into gelatin and gelatin-peptones has been described as a process of hydrolysis, and according to Hofmeister is represented by the following equations:

$$(1)\quad \underset{\text{Collagen}}{C_{102}H_{149}O_{38}N_{31}} + H_2O = \underset{\text{Gelatin}}{C_{102}H_{151}O_{39}N_{31}}$$

$$(2)\quad \underset{\text{Gelatin}}{C_{102}H_{151}O_{39}N_{31}} + 2H_2O = \underset{\text{Semiglutin}}{C_{55}H_{85}O_{22}N_{17}} + \underset{\text{Hemicollin}}{C_{47}H_{70}O_{19}N_{14}}$$

In view of our present knowledge of the final disintegration products of gelatin, it is quite evident that the convenient term "hydrolysis" simply serves to conceal our real ignorance of what actually does occur. Indeed, Emmett and Gies claim that the process is one of molecular rearrangement, and no hydrolysis at all. But even this gives us no definite idea of what happens. With colloidal substances like collagen and gelatin the chemical changes are so closely associated with physical changes that it is doubtful if any line of separation can be drawn between the two.

Hofmeister states that gelatin can be reconverted into collagen by heating it to 130°, which is supposed to indicate that collagen is an *anhydride of gelatin. It is extremely doubtful if collagen is regen*erated under these conditions, the more probable explanation being that, upon driving off the water, the constituent particles of the gelatin approach so close as to form an irreversible gel, thus rendering it insoluble. If only one member of a cumulative protective chain were thus effected, the whole group could become insoluble. (See J. Alexander, "Colloid Chemistry, Theoretical and Applied," Vol. I.)

W. S. Ssadikow (*Kolloidchem. Beihefte,* Vol. 1 [1911]), experimenting with native tendocollagen, finds that the fibrous structure is not lost if it be first dehydrated by alcohol, then dried at room temperature over sulphuric acid, and then heated up to 105°–180°. The collagen remains tough and cannot be ground. Above 180°, the

"intracolloidal" water is driven off, and it browns and becomes brittle. When dried in the presence of its adsorption water, tendocollagen loses its white colour and opacity, and becomes hyaline, just as it does on heating for some time in water, or on immersion in cold weak caustic alkali or strong alkali sulphide. The fibrous form may be restored by soaking the hyaline tendocollagen in cold water, but too drastic a treatment (*e. g.* one month in 2% KOH, or treatment with saturated Na_2S) precludes reversibility.

Thionyltendocollagen is formed on treatment with CS_2; it shows characteristic reactions and is quite resistant to hydrolysis. From 0.12 to 3.05% of sulphur is bound, the amount depending on the previous treatment of the collagen. Ssadikow (*loc. cit.;* also *Kolloid Z.* 1907, **1**, 193) also found that when CS_2 acts on collagen in the presence of alkali, there results what he terms "thiohydratation," the CS_2 combining with the products of alkali hydrolysis, which naturally varies with the concentration and time of action of the alkali. The behaviour of collagen and of gelatin (*vide infra*) with alkali and CS_2 recalls the viscose reaction of Cross and Bevan.

Gelatin[1]

Gelatin does not appear to exist ready-formed in nature, but is produced by the action of hot water on collagen or ossein.

From the evidence at present available, it seems that gelatin is not a definite chemical entity (J. Alexander, "Glue and Gelatin," Chapter 4). Estimates of its "atomic weight" vary materially, as the following table shows:

Shützenberger and Bourgeois	1,836
Paal	900
Wintgren and Krüger	839
Procter and Wilson	768
Berrar	823
Biltz, Bugge and Mehler	5,500 to 31,000
Dorothy Jordan Lloyd	10,300
Dakin	11,800
J. Loeb	12,000 to 25,000
C. R. Smith	about 96,000

R. H. Bogue (*Chem. Met. Eng.*, 1920, **23**, 61) gives analytical results which show the wide percentage variations existing in the several grades of gelatins and glues derived from different sources.

[1] Gelatin has sometimes been called *glutin*, an undesirable synonym, owing to its close resemblance to *gluten*, one of the proteins of wheat.

	Ammonia N	Melanin N	Cystine N	Histidine N	Lysine N	Amino N in filtrate	Arginine N	Non-amino N in filtrate
Hide glues								
Grade 1.....	1.63	0.53	0	1.31	8.17	58.87	13.27	17.00
Grade 2.....	1.89	0.50	0	1.30	8.50	55.17	16.28	15.53
Grade 3.....	3.20	0.74	0	3.19	8.58	55.00	13.70	15.58
Grade 4 ...	2.15	0.53	0	3.31	7.40	57.90	13.72	15.26
Grade 5....	2.44	0.60	0	2.45	8.00	58.02	13.50	15.24
Grade 6. ...	2.49	0.63	trace	1.59	7.22	56.10	12.87	15.20
Bone glues								
Grade 1....	4.43	0.74	0	1.60	7.18	56.90	13.32	16.21
Grade 2....	4.49	1.18	0	0.54	8.23	58.15	12.82	15.18
Grade 3.....	4.57	1.03	0	1.52	7.18	57.30	13.28	15.32
Grade 4.....	4.49	0.82	0	1.44	8.57	57.58	12.74	14.36
Grade 6.....	4.48	0.70	0	1.58	9.42	54.30	13.56	15.90
Grade 9.....	5.04	0.95	trace	4.02	9.13	53.40	13.32	14.54
* Purified hide protein from Grade 1..	1.33	0.78	0	0.82	8.34	60.00	12.61	15.49
* Purified bone protein from Grade 9..	3.57	0.74	trace	2.24	8.60	58.05	10.96	15.47
Isinglass....	3.98	0.68	0	2.33	6.06	58.65	14.20	13.59

* These proteins were obtained by fourfold precipitation with 95 % alcohol.

The Svedberg (*J. Amer. Chem. Soc.*, 1930, **52**, 2897) using the ultra-centrifuge, found that within the region of *p*H 4.6–6.0 there is a very marked aggregation of gelatin molecules, most marked as the isoelectric point is approached, whereas at *p*H 4.0 and 7.5 there is practically no aggregation. Sedimentation equilibrium tests on gelatin solutions at *p*H 4.0 demonstrate their heterogeneity, the molecular weight values drifting from 70,000 at the bottom of the cell to about 10,000 at the top. "It appears probable that the molecular species with molecular weight about 11,000, which is present in the solution to a comparatively large extent, is due to the decomposition products of gelatin. At *p*H 2.5 no sedimentation occurs with a centrifugal force about 100,000 times that of gravity (41,000 r.p.m.), indicating that at this *p*H the gelatin molecules are largely decomposed." Svedberg also reports the partial specific volume of gelatin as 0.682, that of other proteins being about 0.745. Its light absorption, determined by the Judd-Lewis spectrophotometer is small compared with other proteins; the gelatin curve is flat, whereas other proteins show a maximum at 276 mμ.

C. R. Smith of the U. S. Dept. of Agriculture, applied for a public service patent (Serial No. 390, 253, dated June 19th 1920) for preparing ash-free gelatin (see also *J. Amer. Leather Chemists' Assoc.*, Oct., 1922). Sheppard, Elliot and Benedict (*Science*, 1922, **46**,

550) describe an electrolytic method. Dhéré and Gorgolewski (*Compt. rend.*, 1910, **150**, 434) describe a method of making "demineralised" gelatin, which is almost ash-free.

Smith's method, which yields *ash-free isoelectric gelatin* of highest purity is as follows:

Gelatin of highest jelly strength (maximum muta-rotation ratio 2.2) is ground to about 16 mesh and is washed on a filter with cold (0° to 10°) 10% sodium chloride solution containing 5% of concentrated hydrochloric acid per litre, until the washings are free from calcium. The acid is washed out with cold 1% NaCl solution, followed by progressively weaker salt solutions, and finally by distilled or conductivity water until the washings show no trace of chlorine. After dehydrating with cold 90% alcohol, the gelatin is dried.

Smith states that in order to remove all alkali as well as iron and heavy metals, acidulated salt solution must be used. While calcium salts can be removed in about an hour, it takes several hours to remove the hydrochloric acid, the dilute salt solution inhibiting the swelling of the gelatin. As the last traces of acid are being removed, the gelatin (at 15°) shrinks to particles swollen to about 7 volumes.

Ash-free gelatin thus obtained leaves no ash other than traces of sand, if such was in the original. When incinerated with pure sodium carbonate, chlorides, sulphates and phosphates are absent.

Ash-free gelatin swells to from 7 to 8 times its original volume in pure water at 15°; and such swollen pieces melt to yield a clear stable jelly. If a weaker solution be prepared, syneresis occurs, and the jelly becomes cloudy. Thus a 0.5% jelly flocculates into particles (probably swollen to 7 volumes), and the water filtered off shows no trace of gelatin.

Ash-free gelatin forms sols and gels with a minimum stability, and is readily precipitated by alcohol without the presence of electrolytes. Traces of acids or of alkalies increase the osmotic pressure, and prevent precipitation by alcohol, the gelatin thus stabilised or peptised resembling in behaviour the metallic suspensoids. Tap water, containing traces of electrolytes, causes instant precipitation; bivalent and trivalent ions are most effective.

Ultimate analysis of this ash-free gelatin gave:

Carbon	Hydrogen	Nitrogen	Oxygen
50.47	6.75	17.53	25.25
50.56	6.85	17.53	25.04
50.52	6.81	17.53	25.15

See also C. R. Smith, *J. Amer. Chem. Soc.*, 1922, **44**, 214. J. H. Hudson and S. E. Sheppard, *Ind. Eng. Chem.*, 1929, **21**, 263, have made suggestions for the preparation of a *standard gelatin.*

Since gelatin is not a definite chemical entity, it is obviously not correct to speak of "gelatin chloride" and "sodium gelatinate." An extensive literature exists on the nature of the combination between gelatin and acids and alkalies (see J. Loeb, "Proteins and the Theory of Colloidal Behavior"; D. Jordan Lloyd, "Chemistry of the Proteins"; J. Alexander, "Glue and Gelatin"; R. H. Bogue, "The Chemistry and Technology of Gelatin and Glue"). Recently W. D. Bancroft, C. E. Barnett, and B. C. Belden (Colloid Symposium Annual, 1930) determined the combining capacity of gelatin for gaseous ammonia, but there was no evidence of the formation of any definite chemical compound. With gaseous hydrochloric acid, no rise in pressure occurs until after the fixation of about 105–110 mg. HCl per grm. of gelatin. Bancroft believes that this "flat" on the curve shows there are definite chemical compounds, number unknown; beyond this ratio, there is some adsorption. The results show an *equivalent* weight for gelatin of 340± and a molecular weight of some indefinite multiple of this.[1]

For the preparation of pure gelatin the best quality of the commercial article should be soaked for some days in successive quantities of cold distilled water to remove salts and soluble substances, warmed until it dissolves in the water it has absorbed, and the hot solution filtered into strong alcohol (90%). The gelatin is precipitated in white stringy masses, which are collected, redissolved in hot water, and reprecipitated by alcohol. The stronger the alcohol and the lower the temperature, the more persistent the precipitate. The product contains about 0.6% of ash. Commercially, use is made of gelatin solutions containing upwards of 35% of methyl or ethyl alcohol.

The composition of gelatin is approximately: Carbon 50.2, hydrogen 6.7, nitrogen 17.9, oxygen and sulphur 25.0. The following empirical formulæ have been ascribed to gelatin:

[1] Thanks are due to Prof. Bancroft for these as yet unpublished data on acid fixation.

Schutzenberger and Bourgeois	$C_{76}H_{124}O_{29}N_{24}$
Hofmeister	$C_{102}H_{151}O_{39}N_{31}$

When, however, we consider the nature of the products of the hydrolysis of gelatin (see page 147) and also the fact that gelatins from different sources vary in composition, it will be seen that these formulæ tell us very little. For example, the "Hausmann numbers" of gelatin show its nitrogen to be distributed about as follows:[1]

Amide nitrogen	1.61
Amino nitrogen	62.56
Basic nitrogen / Humin nitrogen	35.83

The wide variation in the percentage of sulphur found in gelatin would seem to indicate that the presence of this element is due largely, if not entirely, to the presence of adsorbed impurities. Schlieper found from 0.12 to 0.14% of sulphur in gelatin from bones and ivory, and von Bibra always found very appreciable quantities of sulphur in bone gelatin. Hammarsten found 0.7% of sulphur in fine commercial gelatin yielding 1.74% of ash, and a like amount was found by Chittenden and Solley. It should be borne in mind that commercial gelatin is frequently bleached with sulphurous acid, and then contains sulphites, sometimes in considerable quantity. Modern practise is to oxidise the SO_2 over to sulphates by addition of H_2O_2, or to use the latter as the sole bleach in food gelatin.

Pure gelatin is an amorphous, more or less transparent substance of vitreous appearance, and brittle when dry. It is free from colour, taste and smell.

When heated, gelatin softens without actually melting, swells up and decomposes, with an odour resembling that of burning hair or feathers, leaving a difficulty combustible charcoal, which makes the determination of ash quite difficult.

Air does not affect dry solid gelatin, but when moist or in solution gelatin putrefies with extreme facility, the solution first becoming acid and subsequently ammoniacal. The formation of putrefaction products of acid reaction is characteristic of gelatin. Gelatin gives off or takes up moisture so as to come into an equilibrium with the humidity of its surroundings. In a very moist atmosphere, it may become damp enough to spoil. Bacterial activity is influenced by the *p*H and the amount of contamination with viable organisms,

[1] See "The General Characters of the Proteins," S. B. Schryver, pages 34–35.

and tends to reflect differences in plants and methods. Some jelly powder makers demand a gelatin which, in 2% solution, will show no growth in 72 hours.

When immersed in cold water, gelatin gradually swells up and softens, taking up from 5 to 10 times its weight of water without undergoing solution to any sensible extent. The amount of water absorbed and the rapidity of its absorption, depend upon many factors, chief of which are: (1) Ratio of free surface to volume; hydrogen ion concentration (*p*H); temperature of the system; elastic modulus of the gelatin; ratio of the mass of gelatin present to mass of electrolytes present; the previous history of the gelatin. (See J. Alexander, "Glue and Gelatin," p. 87 *et seq.*) The amount of water absorbed is sufficient to dissolve the gelatin completely upon warming it to 30°.

When gelatin absorbs water, heat is developed and the swollen gelatin occupies a smaller volume than the combined volumes of the original gelatin and the absorbed water. This indicates a compression or condensation of the water within the capillary or molecular interstices of the gelatin. H. G. Bennett ("Animal Proteins" (1921) p. 204) attributes the heat evolved (which amounts to 5.7 calories per grm. of gelatin) to such compression, and predicted from the LeChatelier theorem what was actually found—that gelatin swells best in cold water. Bennett says (p. 209): "The fact of water compression determines the rigidity of the gel, and the changes in this compression of the continuous phase determines the surface tension resultant which hinders swelling and which is one of the two main factors fixing both the rate at which gelatin swells in water, and the final volume attained by the gel." The other factor he refers to is the "lyotrope" (Hofmeister series) effect of salts etc. on the compressibility of water.

Pure gelatin dissolves readily in boiling water, solution being greatly facilitated by preliminary swelling in cold water and by agitation. If powdered gelatin is to be dissolved directly in hot water, it should be added slowly, with vigorous stirring, to prevent formation of lumps which are difficultly soluble. The reaction of the solution depends on the *p*H of the original gelatin. If the hot aqueous solution be very dilute, it remains unchanged upon cooling, but if somewhat stronger it becomes viscous. When the proportion of gelatin exceeds about 1%, the solution sets to a jelly upon cooling,

the resiliency or consistency of the jelly formed increasing with the concentration of the liquid, but being also dependent on the origin, test, and purity of the gelatin. The jelly liquefies again completely at about 40° C.; repeated heating and cooling destroy the property of gelatinising, and long-continued boiling at ordinary pressure has the same effect. The gelatinising power is lost speedily by heating the solution under increased pressure at 140°.

If potassium dichromate be added to a solution of gelatin in hot water, the jelly which forms on cooling becomes insoluble in warm water after exposure to light. This fact is largely used in photo-lithography. (Collotype).[1]

Solutions of gelatin are eminently undiffusible; in fact, the term "colloid"—literally "glue-like"—was for this reason invented by Thomas Graham to describe such substances. From the modern colloid-chemical point of view, a warm liquid solution of gelatin in water would be classed as a *hydrosol*, or for brevity a *sol*. As pointed out by Procter, the cold solution or jelly can hardly be called a *gel;* in fact, the term "solid hydrosol" proposed by Lottermoser appears to be more applicable. In the ultra-microscope, a hot solution of pure gelatin shows a clear field, the few ultra-microns and slight Tyndall effect being probably due to traces of impurities. According to R. Zsigmondy a 0.2% solution of gelatin 2 days old appears heterogeneous in the ultramicroscope. The fluid is entirely filled with small whitish particles at the limit of visibility (about 5 $m\mu$), and with these were visible larger hydrogel particles. In a 0.01% solution the distance between the free moving particles was less than 1 μ. A solution of gelatin prepared at the boiling-point showed, however, a homogeneous light-cone in the Siedentopf-Zsigmondy ultra-microscope.

W. Menz (*Z. physik. Chem.*, 1909, **66**, 129) followed the formation of gelatin gels ultramicroscopically. On cooling a 0.5% solution, the field becomes luminous, and submicrons appear suddenly and aggregate to form flocks. That gelatin jellies may undergo a slow

[1] The exact *modus operandi* varies materially according to circumstances. Broadly speaking, it consists in obtaining a sensitive film by pouring a solution of dichromated gelatin on to a glass or metal plate. This is exposed under a photographic negative, and then treated with cold water. The gelatin in those parts of the film protected from the light by the opaque parts of the negative, absorb the most water and swell up above those parts affected by the light, which thus form depressions, whose depth increases with the amount of light transmitted. When inked over, the ink enters the depressions, but is repelled by the moist high lights, and, on printing, a reproduction of the photograph is obtained. Another method is to treat the exposed plate with warm water, which dissolves the soluble protected parts, thus forming a reproduction of the photograph in relief. This may be hardened with alum or formaldehyde and used in a printing press, or the impression may be transferred to a lithographic stone.

annealing or hysteresis on ageing, is evident from the observations of F. Stoffel (*Inaug. Disser.*, Zurich, 1908) that the difference in permeability to a diffusing substance exhibited by gels chilled at different speeds, become equalised on standing for several days at room temperature.

W. Bachman[1] has investigated the ultra-microscopic structure of gelatin jellies with the slit and cardioid ultra-microscopes.

In the case of 1–2% solutions of gelatin, optically homogeneous except for dust particles and similar impurities, the course of gelatinisation exhibited six different phases: (1) a homogeneous light-cone, linearly polarised; (2) a scintillating motion, which gradually resolved into (3) a dense mass of submicrons with a translatory motion; (4) diminution of the Brownian movement of the optical impurities of the gelatin solution; (5) diminution of the translatory motion of the gelatin submicrons and oscillatory motion about a mean position of equilibrium; (6) a differentiated gel with quiescent "jelly elements" (*i. e.*, the smallest structural elements detectable by ultra-microscopic means), which because of their strong linear polarisation of light, are probably aggregations of submicrons and amicrons. The mass of the gel is resolved into ultra-microscopic and microscopic elements, which, in the order of their magnitude, lie near the limit of the power of resolvability of the microscope; these elements may be microscopic in one dimension and ultra-microscopic in another.

Increasing concentration renders the ultra-microscopic heterogeneity of the jellies less and less distinct; jellies of 7–10% concentration appear homogeneous, but the strong polarisation of the diffuse light cone indicates that they are actually heterogeneous and probably composed of extremely fine particles formed into larger jelly elements. The more concentrated the jelly, the closer together these "jelly elements" would be, and the smaller would be their difference in composition and therefore in refractive power between the two phases, so that at a certain concentration the two phases would be indistinguishable even with the ultra-microscope, *i. e.*, the jelly would appear homogeneous. In the case of gelatin solutions of such small concentration that they do not gelatinise (0.5%) flocks are gradually formed owing to the aggregation of isolated "jelly elements" or of submicrons.

[1] *Z. anorg. Chem.*, 1911, 73, 125–172.

The observations in all cases indicate a very much finer structure for jellies than that described by Bütschli. Jellies treated with alcohol or chromic acid showed under the microscope the honeycomb structure of Bütschli, but the structure revealed by the ultramicroscope was a granular ("globulitic") one; and it was found that the alcohol (or chromic acid) caused coagulation and shrinking (dehydration) and that these are the cause of the honeycomb structure of Bütschli, the true structure of the jelly being much finer.[1]

P. Krishnamurti (*Indian Journal of Physics*, 1928, Vol. 3, Part 3, p. 307) studied the nature of gelatin, dextrin, and sodium oleate solutions by X-ray diffraction methods, and concludes: "From osmotic pressure measurements and from the evidence of the ultramicroscope, we can say that the ultimate particles of gelatin are of very large size when compared to ordinary molecular dimensions. But it has not been clear till now whether it is molecularly dispersed or whether it forms groups which are only aggregations of simpler molecules. X-ray evidence shows that a gelatin solution consists of molecules of gelatin, the molecules themselves being of very large size, probably formed from simpler molecules by chemical union, and hence not breaking down appreciably on dilution. This case is quite different from that of sodium oleate, which forms groups containing several molecules of simple sodium oleate." J. G. McNally and W. Vanselow (*J. Amer. Chem. Soc.*, 1930, **52**, 3846) found that a decrease in the viscosity of gelatin solutions caused by hydrolytic dispersion of micellular structures is accompanied by a decrease in the intensity in *fluorescence* to light waves 365 mμ in length. Subsequent chemical changes cause an increase in fluorescence.

Gelatin is a typical reversible, (Hardy, Zsigmondy), emulsion, (Wolfgang Ostwald), or hydrophile-colloid (Perrin). When dried out at temperatures under 100°, it can be redissolved, and this operation repeated again and again. It is also a most powerful protective colloid (Schutz-kolloid) showing the lowest gold-figure on Zsigmondy's table.[2] For this reason even traces of gelatin may act as a "colloidiser," and cause great difficulty for the analyst by entirely inhibiting the formation of precipitates, or else by causing the precipitate to form in such a finely subdivided or colloidal condition, that it will pass through the finest filter paper.[3]

[1] See also R. Zsigmondy, *Z. anorg. Chem.*, 1911, 71, 356–377.

[2] See R. Zsigmondy, "Colloids and the Ultra-microscope," page 81; Alexander," Glue and Gelatin," p. 142, also p. 146.

[3] See J. Alexander, *J. Soc. Chem. Ind.*, 1909, **28**, 280; also "Colloid Chemistry," Vol. I, article by Prof. H. Bassett.

Gelatin exhibits a strong lævorotation, which varies with the temperature (muta-rotation). See p. 142.

Reactions of Gelatin

Gelatin is practically insoluble in ice-cold water containing 10% of alcohol. This fact is employed for its determination. In absolute alcohol, gelatin is insoluble at any temperature, and on adding excess of alcohol to its aqueous solution, the gelatin is thrown down as a white, coherent, elastic mass. No true coagulation occurs, for the precipitate has all the characters of the original gelatin, swelling up in cold water and dissolving on heating.

Gelatin is wholly insoluble in absolute alcohol, ether, chloroform, benzene, carbon disulphide, and fixed and volatile oils.

Selenium oxychloride readily dissolves gelatin and glue in the cold (V. Lenher, *J. Amer. Chem. Soc.*, 1921, **43**, 29). They may also be held in fluid state at room temperature by urea, zinc, magnesium and calcium chlorides, sodium iodide, sodium and calcium nitrates, naphthalene sulphonate, etc.

Strong acetic acid dissolves gelatin to form a solution which does not gelatinise on cooling, but yet possesses powerful adhesive properties. "Coagulin" and similar commercial preparations are of this nature. Dilute nitric acid forms a similar product soluble in cold water ("soluble glue"; compare page 126). When heated with strong nitric acid, gelatin is destroyed, with formation of oxalic acid and other products.

Boiling with hydrolysing agents converts gelatin into a mixture of gelatin-peptones or gelatones (page 126).

On dry distillation, gelatin gives a highly offensive oil (Dippel's oil; bone oil), containing a considerable proportion of pyridine bases, together with ammonia, permanent gases, and other ordinary products of the decomposition by heat of nitrogenised organic matters.

On adding gallotannic acid to an aqueous solution of gelatin, a white or buff-coloured precipitate is formed. A solution of gelatin in 5,000 parts of water is at once rendered turbid by gallotannic acid or other variety of tannin. Nevertheless, the precipitate is not wholly insoluble in pure water, especially if hot, but is quite insoluble in presence of excess of tannin.

The reaction of gelatin with tannin is utilised for the detection of both substances, and is employed for the assay of tannin matters.

(See Vol. V, page 174.) It is also utilised for tanning, the gelatinoids of the skin being thereby converted into leather.

To replace hide powder in tannin analysis, E. Giusiana[1] proposes the use of fish glue or gelatin previously tanned in a basic chrome solution prepared from 100 grm. of chrome alum crystals, 1,000 c.c. of water and 15 grm. of crystallised sodium carbonate. After soaking for 24 hours in this solution, thin sheets of gelatin are completely tanned and are quite insoluble in hot water. They are washed, neutralised in 1% ammonia, again thoroughly washed and dried between filter papers. The tanned gelatin may be kept indefinitely in distilled water. For 100 c.c. tannin solution the equivalent of 5 grm. of dry gelatin is used. The shakings, filtrations, etc., are the same as in the hide powder process.

It is misleading to speak of the combination between tannin and gelatin at *tannate of gelatin*, for the ratio in which these substances combine varies greatly with the conditions of the experiment. The precipitation of gelatin with tannin is a typical instance of the mutual coagulation of oppositely charged colloids. In aqueous solution tannin is a positively charged colloid (negatively conducted). Gelatin, on the other hand, is amphoteric; and as Ricevuto has pointed out (*Koll. Zeit.*, 1908, **3**, 114), it is not precipitated by tannin unless it is in the negative condition (positively conducted), *i. e.*, carefully dialysed gelatin is not precipitated by tannin. The so-called *tannate of gelatin* is therefore a variable colloid or absorption compound, and, as von Schroeder has shown, the precipitation of gelatin by tannin follows the adsorption isotherm. (*Kolloidchem. Beihefte*, **1**, No. 1.)

Basic chromium solutions, as well as bichromates, render gelatin insoluble; the effects of chromium salts form the basis of "chrome tanning" and of several engraving and photographic reproduction processes. (See *e. g.* Eder, "Reaktionen der Chromsäure und der Chromate auf organische Substanzen etc.," 1878.) L. Meunier and A. Seyewetz (*Collegium*, 1908, No. 313, p. 195) report having precipitated gelatin with the following organic compounds: phenol, resorcinol, orcinol, hydroquinone, pyrocatechin, pyrogallic acid, *p*-amidophenol, chlorophenol, monochlorhydroquinone (durol), R acid (disulpho-β naphthol 2, 3.6), G acid (disulpho-β naphthol 2, 6.8), S acid (monosulpho-β naphthol 2.6).

[1] *J. Amer. Leather Chem. Assoc.*, 1913, **8**, 143.

Thomas Graham (*Trans. Roy. Soc.*, 1861, **151**, 206) found that colloidal silicic acid reacts with gelatin. Alums, iron salts, uranic salts, auric chloride, and some copper, silver and lead salts are fixed by gelatin, often tanning it powerfully. Even barium chloride is partially hydrolysed by it, and if a red solution of thiocyanate of iron be added dropwise to a 10% gelatin solution, the ruby red precipitate soon exhibits the rusty brown colour of ferric hydroxide. (For further details see J. Alexander, "Glue and Gelatin," Chapter 9 (1923).)

A. E. Jones (*Analyst*, 1927, **52**, 275) tested the behaviour of 73 substances (non-tannins) towards gelatin solutions and found that the following 18 give a precipitate: (a) *in 1% solutions*—gallic acid, *β*-resorcylic acid, haematoxylin, brazilin, picric acid, ethyl gallate, *m*-hydroxybenzoic acid, hydroxyhydroquinone, and maclurin; (b) *only in saturated solutions*—phenol, resorcinol, catechol, phloroglucinol, pyrogallol, methyl gallate, guaiacol, and protocatechuic acid. "From these observations it is evident that a positive gelatin test is no specific indication of the presence of a tannin."

Gelatin is not thrown down from its aqueous solution by the ordinary mineral or organic acids, by alkalies,[1] or by most other metallic salts. Phosphomolybdic and phosphotungstic acids precipitate it, as does mercuric chloride (if used in excess); but no precipitate is produced by alum, ferric chloride or sulphate, salts of copper, or by neutral or basic lead acetate. Saturation of its aqueous solution by ammonium sulphate, magnesium sulphate, or zinc sulphate completely precipitates gelatin.

On adding a saturated aqueous solution of picric acid to a cold aqueous solution of gelatin, a precipitate is produced which redissolves on shaking. On gradually continuing the addition of picric acid, the precipitate becomes permanent; but dissolves on heating, and reappears as the solution cools. The precipitate coagulates on shaking, producing a yellow sticky mass, and leaving the liquid nearly clear (Allen and Tankard).

Upon exposure to light a solution of gelatin containing auric chloride develops a ruby red colour, due to the formation of colloidal gold.

Platinic chloride solution reacts with gelatin in much the same way as picric acid, except that the precipitate does not appear to be so readily soluble in excess of gelatin.

[1] Addition of sodium hydroxide or ammonia to a solution of commercial gelatin often produces a considerable precipitate of calcium phosphate.

Platinic sulphate solution also gives a precipitate with solutions of gelatin, which rapidly coagulates on standing, even without shaking. In other respects, the reaction of gelatin with platinic sulphate is similar to its behaviour with the chloride.[1]

L. Crismer recommends an acid solution of chromic acid as a precipitant for gelatin (compare page 134).

In the biuret test (Vol. VIII, p. 649) gelatin gives a violet coloration. No precipitation of cuprous oxide occurs on boiling the liquid.

When a current of chlorine is passed through a solution of gelatin of about 1% strength, the liquid remains clear for a time, but subsequently froths strongly, each bubble becoming encased in a white pellicle. When the chlorine is in excess, as indicated by the yellow colour of the liquid, the frothing subsides, the liquid becomes clear, and the whole of the gelatin is thrown down as a white granular precipitate. When this is thoroughly washed with cold water and dried *in vacuo* over sulphuric acid, the substance is obtained as a pale yellowish-white powder, which is odourless, tasteless, and imputrescible, and insoluble in water or alcohol, but soluble in alkalies.[2]

According to Cross, Bevan and Briggs (*J. Soc. Chem. Ind.*, 1908, **27**, 260), moist gelatin when spread out very thin by immersing cotton yarn in its solution, combines with 15.4% of chlorine gas, forming what they call a gelatin chloramine. Different qualities of gelatin give approximately identical results. This chloramine is sensitive to "antichlors," and when treated with sulphurous acid reverts to the original condition. These authors have used the chloramine reaction as the basis of a method for estimating gelatin in tub-sized papers.

Contrary to the general statement, that bromine and iodine give no compound with gelatin similar to that yielded with chlorine, Allen in collaboration with A. B. Searle (*Analyst*, 1897, page 258) proved bromine to act in a very similar manner to chlorine, and this observa-

[1] According to E. Davy, platinic sulphate precipitates gelatin in the form of brown, viscous flakes, which blacken on the filter and are afterward easily pulverised. He regards the reaction as an infallible test for gelatin, applicable even in presence of albumin and in solutions so dilute as to give no indication with tannin. The author Allen has been unable to confirm Davy's observation.

[2] The observations described in the text are those of Rideal and Stewart (*Analyst*, 1897, page 228). The reaction of chlorine on solutions of gelatin was first described by Mülder in 1840, and was regarded by him as an argument in favour of his protein theory, the precipitate itself being termed protein-chlorous acid (*Berzelius' Jahresber.*, **19**, 734; *Jour. f. Chem.*, **44**, 489). Considerable controversy occurred as to the chemical nature of the compound, but all agreed as to its properties, its constancy of composition, and its insolubility. The proportion of nitrogen found in the compound by the early observers is in close agreement with the percentage found by Rideal and Stewart in specimens prepared by them and analysed after drying at 80°.

tion has been independently made and extended to iodine by Hopkins and Brook (*J. Physiol.*, **22**, 184). The method of operating employed by Allen is that described in Vol. IX, p. 516, but the process as applied to gelatin is not at present capable of successful employment.[1]

Gelatin forms an insoluble compound with formaldehyde, the maximum amount permanently fixed, according to Lumière and Seyewetz (*Bull. Soc. Chim.*, 1906, **35**, 872; *J. Soc. Chem. Ind.*, 1906, **25**, 1058) being between 4.0 and 4.8%, when 10% formaldehyde solution acts upon dry gelatin. Formo-gelatin see p. 153 is decomposed by repeated treatment with boiling water, by cold 15% hydrochloric acid, and by heating to 110°. Lumière and Seyewetz have also investigated the insoluble compound formed between gelatin and quinone (*Bull. Soc. Chim.* 1907, [iv], **1**, 428–431; *J. Soc. Chem. Ind.*, 1907, **26**, 703). They find that 100 grm. of gelatin are rendered insoluble by 1 grm. of quinone, the time varying with the concentration of the solutions. The gelatin product thus obtained has a pink colour and swells in cold water, becoming elastic; but it becomes brittle again upon drying. Prolonged treatment with boiling water, or with solutions of ammonia or alkali carbonates do not affect it, but it is gradually decomposed by solutions of acids or alkali hydroxides.

The polariscopic examination of gelatin is of particular interest, for C. R. Smith states (*J. Amer. Chem. Soc.*, 1919, **41**, 135) that, so far as known, gelatin is the only protein exhibiting ***muta-rotation***, but loses this power when its gelatinising power is completely destroyed by heating its solution. At 35° C. the specific rotatory power, $[\alpha]_D$, of the purest gelatin then commercially available was $-141°$, on a moisture- and ash-free basis. When cooled to 15° or lower, $[\alpha]_D$ was found to be $-313°$, on a moisture- and ash-free basis, the mutation ratio being 2.21 to 1. Above 35° C. it requires much more alcohol to precipitate the gelatin—about 45–50%.

Detection of Gelatin.—The recognition of gelatin in animal fluids is attended with some difficulty. The reaction with tannin is fairly delicate, but is also given by all the proteins. The behaviour of gelatin with picric acid (page 142) is peculiar, and may be occa-

[1] Experiments made under Allen's direction by A. R. Tankard, on various specimens of commercial gelatin and glue, yielded results which at present are incapable of interpretation. The completeness of the precipitation of gelatin by bromine-water is affected by conditions not at present understood. In some cases the precipitation was very complete, while in other experiments, in which the conditions were but very slightly varied, much nitrogen remained unprecipitated. "Previous history" of colloids affects their structure and hence their ability to form adsorption compounds.

sionally useful for its recognition. As a rule, the property of gelatinising on cooling is a test from which the presence of gelatin in a complex animal liquid can be safely inferred.

C. R. Smith (*J. Ind. Eng. Chem.*, 1920, **12**, 878), in suggesting a polariscopic method for detecting gelatin, says: "A progressive increase in aevorotation (or muta-rotation) obtained from a solution cooled quickly from 35° C., accompanied by the production of a jelly after a change of approximately 4.7° Ventzke, is very positive proof of the presence of gelatin in any solution concentrated enough to jelly."

H. C. Gore (*Ind. Eng. Chem., Analytical Ed.*, 1929, **1**, 203) proposes the progressive decline in the muta-rotation of gelatin solutions undergoing proteolysis by enzymes (papain, pepsin, pancreatin, and the proteolytic enzyme of malt) as a new method of measuring proteolytic activity, which is thus analogous to the Lintner method for determining and expressing numerically diastatic power. Five samples of papain assayed by this method varied in proteolytic power by about 100%.

Trillat (*Comptes rend.*, 1898, **127**, 724; *J. Soc. Chem. Ind.*, 1898, **18**, 78) has proposed to estimate gelatin in gums and alimentary substances by weighing the precipitate produced by formaldehyde. Henzold (*Z. offentl. Chem.*, 1900, **6**, 292; *J. Soc. Chem. Ind.*, 1900, **19**, 1042) proposed the following method: The material is boiled with water, and the filtrate boiled with an excess of 10% potassium dichromate solution. After cooling, if gelatin be present, a few drops of concentrated sulphuric acid produce a white flocculent precipitate, which gradually agglomerates at the bottom of the vessel.

The U. S. Department of Agriculture[1] use Stokes' picric-acid method (*Analyst*, 1907, **32**, 320) for detecting the presence of gelatin in cream or ice-cream. The procedure is as follows: Dissolve 5 grm. of mercury in 10 grm. of nitric acid (sp. gr. 1.42) and dilute to 25 times its bulk. To 10 c.c. of this solution add 10 c.c. of cream and 20 c.c. of water, in order to precipitate all proteins except gelatin. If gelatin is present, the filtrate will give an immediate yellow precipitate with an equal part of a saturated aqueous solution of picric acid.

G. E. Patrick (*U. S. Dept. Agr., Bull.* 116, p. 24) pointed out that sour cream containing no gelatin frequently gives the above reaction,

[1] *Dept. Agric. Bur. Chem. Bull.*, 107, rev., page 121, 1910.

because of the presence of "pseudo-gelatin" decomposition products. A. Seidenberg (*J. Ind. Eng. Chem.*, 1913, **5**, 927) found that the two precipitates may be differentiated by the fact that while both precipitates are soluble on heating in slightly acid solutions, only the "gelatin picrate" is soluble in hot neutral water alone. The picric acid precipitate from sour cream appears to be entirely insoluble in hot water, after all the picric or other acid has been removed. To accomplish this, the picric acid precipitate is shaken vigorously to cause it to coalesce, and after standing, most of the liquid can be siphoned off. The precipitate is washed on a filter with water containing 2 or 3 drops of ammonium hydroxide to 100 c.c., until the washings are slightly alkaline to litmus; it is then boiled with 10–20 c.c. of distilled water and filtered hot into a test-tube. If gelatin is present, the cooled filtrate will give a decided precipitate with an equal volume of picric acid solution. By this method it is possible to detect 0.5% of gelatin in 20–25 c.c. of sour cream.

For the *detection of glue* in sizing materials E. Schmidt (*Farber Ztg.*, **24**, 97–98) uses ammonium molybdate and a modified Nessler's solution, which he finds more sensitive than the biuret test or precipitation with tannin. The special Nessler solution is prepared by making ordinary Nessler solution slightly acid with sulphuric acid, and filtering to remove the red precipitate. The clear yellow filtrate is a sensitive test for glue even in the presence of ammonium salts.

Both of these reagents give a faint turbidity with a solution containing 0.00001 grm. glue in 5 c.c. water. Norgine and albumin interfere with the test, and must be removed by adding dilute nitric acid to the solution, freed from fat, and then filtering.

To separate gelatin from an aqueous solution containing proteins, albumoses, peptones, mucin and casein, Berrár (*Biochem. Z.*, 1912, **47**, 189–214) adds to the solution about 2 volumes of a mixture of 1 part of a saturated aqueous solution of picric acid with 4 parts of 96% alcohol. The gelatin alone remains in solution, and after filtration may be quantitatively precipitated by adding about 1½ volumes of saturated aqueous picric acid solution and allowing the mixture to stand for 12 hours at 10°. By this means he accurately estimated gelatin mixed with milk and egg albumin. The gelatin picric-acid precipitate was washed with a solution of potassium mercuric iodide until the washings were no longer yellow in colour;

the quantity of gelatin in the residue was found by estimating the nitrogen content.

For the qualitative detection of gelatin in the presence of proteins, the filtrate from the alcohol-aqueous picric-acid solution, is tested *with picric acid solution, a yellowish-white opalescence being* formed even at a concentration of 1 in 100,000. The opalescence is best detected by a "ring-test."

Berrár also found that the solubility of purified gelatin in cold water is 0.56, 0.60 and 0.62% at temperatures of 17°, 18° and 19°, respectively. These concentrations approximate to the minimum required for the formation of a jelly; thus a 0.7% solution in hot water sets to a thin jelly on cooling. If a saturated solution, formed in the cold, remains in contact with pieces of gelatin, a partial separation of the dissolved substance occurs upon further cooling, but no gelatinisation. Neither alcohol nor metaphosphoric acid *precipitates gelatin completely from* aqueous solutions. The same is true of picric acid at ordinary temperature, but at 8° complete precipitation occurs on adding an equal volume of saturated aqueous picric acid solution. When gelatin is completely precipitated by acid reagents, *e. g.*, by a solution containing picric acid and potassium mercuric iodide (the precipitate may in this case be safely washed with water) the gelatin and acid combine in definite proportions, 1 molecular equivalent of acid combining with a quantity of gelatin containing 10 atoms of nitrogen. On the assumption that 1 molecular equivalent of acid unites with 1 molecule of gelatin, the molecular weight of the latter must be 823 (Paal found 900).

The nitrogen content of picric acid or of precipitates containing this compound and proteins or gelatin, may be estimated accurately by a modification of the Kjeldahl method. The material is treated in a Kjeldahl flask, with 0.5–1 grm. of iron filings, 5 c.c. of glacial acetic acid, 20 c.c. of concentrated sulphuric and some copper sulphate; the mixture is heated in the ordinary way, and the subsequent operations carried out as usual.

Remington and McRoberts (*Ind. Eng. Chem.*, 1927, **19**, 267) suggest a modification of Ferris' method (*J. Dairy Science*, 1922, **5**, 555) for the determination of gelatin in ice cream. They use a smaller sample, dilution by weight instead of by volume, control of casein precipitation by a hematoxylin indicator, the use of alum to assure complete precipitation of the gelatin, and the correction of the N

found for that from the milk entering the filtrate. After precipitating the casein, 100 c.c. of the filtrate is treated with 3 c.c. of a saturated solution of potassium alum and 200 c.c. of 95% alcohol. The precipitate, separated on a Büchner funnel, is redissolved, made up to 100 c.c. and a polarisation is made at 35°. Or a nitrogen determination may be made on 25 c.c. Factors are given to fix the percentage of gelatin in either case. For exact details the original should be consulted.

For the detection of gelatin in preserves, A. Desmoulière (*Ann. Chim. anal. appl.*, **7**, 201; *J. Soc. Chem. Ind.*, 1902, **21**, 1157) treats 20 grm. of the preserve with 100 c.c. of 90% alcohol, added gradually, and allows the mixture to stand for 2–3 hours.[1] The liquid is carefully decanted and the residue dissolved in hot water. In the presence of gelatin, the solution gives precipitates with tannin and picric acid, and evolves ammonia when treated with calcium oxide (quicklime).

Dürbeck (*Z. Nahr. Genussmittel*, **27**, 801) states that a solution of thionin, a thiazin dye, will differentiate between agar and gelatin in sausage, the former giving a violet colour, and the latter a deep blue.[2]

Gelatin is distinguished from albumin and its allies by not yielding a precipitate with potassium ferrocyanide or ferricyanide. Reactions distinguishing gelatin from chondrin and mucin are described on pp. 172, 173.

Gelatin is very readily digested; and the fact that "calves'-foot" and similar jellies have for years been recommended by physicians as a food for invalids and convalescents, indicates that it has considerable food value. Guérard and other French investigators believed gelatin to be a very nourishing food. Voit, however, showed by feeding experiments that gelatin does not build up the body, but is broken up and used in place of the circulating albumin, which is thereby economised. It thus spares both the circulating and the organised albumin, and accomplishes the same results as the carbohydrates and fats, only to a more intense degree.[3]

Gelatin also is a great aid in the digestion and absorption of other foods. An important instance of its value in this respect may be

[1] Since gelatin is slightly soluble in 90% alcohol (*J. Soc. Chem. Ind.*, 1904, **23**, 380), somewhat stronger alcohol should be used.

[2] If the sausage contains gelatin-forming material, this may yield gelatin on cooking.

[3] A. Jacobi, "Intestinal Diseases of Infancy and Childhood," 1889, Vol. I, page 64. See also J. R. Murlin, *Amer. J. Physiol.*, 1907, **19**, 285; *Chem. Abs.*, 1907, 2392.

seen in the case of milk. The addition of gelatin to cows' milk inhibits the coagulation of the casein by acid or by rennin. In coagulating, the casein mechanically entraps the milk fat, forming a greasy curd very difficult to digest, and in preventing its coagulation, the gelatin (which acts here as a protective colloid) furthers the digestion of both the casein and the fat.[1] Recently C. A. Herter pointed out that gelatin is especially valuable in cases of bacterial infection of the intestinal tract, because it contains neither the trytophane nor the tyrosine nuclei and cannot yield indole, skatole and other objectionable products.[2] Since tyrosine and tryptophane are essential amino acids, gelatin will not serve as the *sole* protein in any diet.

The powerful colloidal protective effect of gelatin may be seen from the following table of "gold numbers"[3] based on the results of Zsigmondy and of Schryver:

Substance	Gold number
Gelatin	0.005– 0.01
Glue	0.005– 0.01
Isinglass	0.01 – 0.02
Casein (ammonia solu.)	0.01
Ovoglobulin	0.02 – 0.05
Ovomucoid	0.04 – 0.08
Glycoprotein	0.05 – 0.1
Amorphous egg albumin	0.03 – 0.06
Crystallised egg albumin	2.0 – 8.0
Fresh egg white	0.08 – 0.15
Gum arabic	0.15 – 4.0
Gum tragacanth	2.0 ±
Dextrin	6.0 –20.0
Starches	5.0 –25.0
Sodium oleate, at 100°	0.01
Sodium oleate, at 60°	10.0
Cane sugar	∞
Urea	∞
Deutero-albumose	∞

F. A. Elliott and S. E. Sheppard, *J. Ind. Eng. Chem.*, 1921, **13**, 699, investigated the gold numbers of commercial gelatins, and found that (as Zsigmondy found with sodium oleate) the degree of dispersion is a big factor. Thus the gold number decreases as concentration decreases, but rises on ageing.

[1] See J. Alexander, *Z. Chem. Ind. Kolloide*, 1909, **5**, 101; 1910, **6**, 197; *J. Amer. Chem. Soc.*, 1910, **32**, 680; Alexander and Bullowa, *Arch. Pediatrics*, 1910.

[2] See C. A. Herter, "Infantilism," 1908, page 96. The protective action of the gelatin by preventing the formation of large persistent clumps, prevents bacterial decomposition on the interior of such clumps.

[3] For the gold number test and preparation of the solutions, see R. Zsigmondy, *Z. anal. Chem.*, 1901, **40**, 697, or "Colloids and the Ultramicroscope" (1909); or J. Alexander, "Glue and Gelatin" (1923), p. 141.

Cleavage Products of Gelatin.—Gelatoses, Gelatin-peptones or Gelatones, Peptides, Amino-acids.

In common with other proteins, gelatin is split up or decomposed by acids, alkalies, proteoclastic enzymes and bacteria, the nature of the degradation products varying with the conditions of the experiment. Under the action of these agents gelatin loses its characteristic property of forming a jelly when its hot, tolerably concentrated, aqueous solution is cooled. The products formed by the hydrolysis of gelatin are analogous to those produced by the hydrolysis of other proteins (Vol. VIII, 623) and may be classified as follows:

1. Metaproteins = { acid gelatin
alkali gelatin
2. Proteoses[1] = gelatoses
3. Peptones = gelatones
4. Polypeptides or peptides } possess protein properties.
5. Amino-acids.

These groups are not at present very sharply defined, but, in general, gelatones differ from gelatoses in that they are more diffusible, not precipitated by saturation with ammonium sulphate, and give no reaction with potassium ferrocyanide and acetic acid, potassio-mercuric iodide and hydrochloric acid, picric acid, and trichloroacetic acid. Peptones are now known to consist of peptides or mixtures of peptides, but the term peptide is at present reserved for those possessing a recognised structure.

Gelatoses are divided into *protogelatoses*, which are precipitated upon the addition of an equal volume of cold-saturated ammonium sulphate, and *deuterogelatoses*, which are precipitated only upon saturation with this salt. Two distinct gelatones are recognised by Hofmeister, who terms them, respectively, semiglutin and hemicollin (see page 127). Semiglutin is sparingly soluble in 70 to 80% alcohol and is precipitated by platinic chloride, whereas hemicollin is soluble in 70 to 80% alcohol and is not precipitated by platinic chloride.

C. Paal (*Ber.*, **25**, 1202) has shown that when gelatones[2] are formed by the action of dilute mineral acids on gelatin they unite with the

[1] Proteoses are sometimes called "albumoses," a term that should be reserved for the proteose of albumin.

[2] P. A. Levene (*Z. physiol. Chem.*, 1904, 41, 10) prepared gelatin peptone by dissolving gelatin in 10 times its weight of 0.5 % sodium carbonate, adding 0.1 % of trypsin with a little toluene as preservative. After several month's standing the solution was concentrated, and the traces of proteose still remaining removed with ammonium sulphate, which was then dialyzed out. The gelatone was precipitated from the acidified solution by phosphotungstic acid, and the precipitate washed with boiling water to remove hexone bases, until the wash water gave no precipitate upon standing. The residue, which formed a hard stone-like mass on cooling, was dissolved by rubbing in a little ammonia, the sul-

acid to form salts, which are not only soluble in water, but, unlike the free gelatones, in ethyl and methyl alcohols. For their preparation, Paal warms 100 parts of the purest commercial gelatin on the water-bath with 160 parts of water and 40 of concentrated hydrochloric acid, until a sample of the product is completely soluble in a large quantity of absolute alcohol. The whole is then poured into 4 or 5 volumes of absolute alcohol, the precipitated salts filtered off, the filtrate treated with ether, the precipitate redissolved in alcohol, and the resulting solution evaporated under reduced pressure. The gelatone hydrochloride thus obtained forms a brittle, white, vesicular mass, which is readily soluble in water, methyl alcohol, ethyl alcohol, and acetic acid; but is insoluble in ether, benzene, or carbon disulphide. It is very hygroscopic, is unaltered at 130°, and gives a violet coloration in the biuret test. It is lævorotatory, the value of $[\alpha]_D$ in aqueous solution being about −60°. The proportion of hydrochloric acid found in different preparations ranged from 10.5 to 12.5%, while the ash was only about 0.5%. When stronger hydrochloric acid was employed, or the heating continued longer, compounds containing a higher percentage of acid were obtained. This fact tends to show that the products are a mixture of compounds representing different degrees of peptonisation. The hydrochlorides containing high proportions of acid are more diffusible and soluble than those containing small percentages of hydrochloric acid. Thus, by dialysing a salt containing 10.56% of acid, Paal obtained from the diffusate a compound containing 14.19% of hydrochloric acid, whereas the residue contained only 5.79% and was insoluble in absolute alcohol.[1]

A similar separation was effected by treating the alcoholic solution with mercuric chloride, whereby two mercurichlorides were obtained.

phuric and phosphotungstic acids removed, and the ammonia evaporated. The gelatone was further purified by washing with absolute alcohol and ether, the former of which dissolved considerable of it. The peptone insoluble in alcohol was a white very hygroscopic powder.

The results of the analysis of Levene's peptone, as compared with those prepared by Siegfried, and by P. Tartarinoff (*Compt. rend.*, 97, 713), are given in the following table:

	C	H	N	Difference
Levene	45.96	6.71	17.93	29.40
Siegfried	46.76	6.23	17.32	29.69
Tartarinoff (average)	49.76	7.13	12.63	30.48

[1] It was found that only those salts containing 10 % or upward of hydrochloric acid were soluble in absolute alcohol, and those fractions which were only just dissolved by ethyl alcohol were much less soluble in propyl alcohol, and insoluble in amyl alcohol; while the salts with less than 10 % of acid were all readily soluble in methyl alcohol, and dissolved also in the ethyl alcohol solutions of the salts containing more acid.

One of these separated out at once, and the second on addition of ether.

On warming gelatin with a weaker acid than was used in the foregoing experiments a salt was obtained which contained 6.85% of hydrochloric acid, and was insoluble in ethyl alcohol, but soluble in methyl alcohol; while the product obtained by means of pepsin and very dilute hydrochloric acid was not completely soluble in cold methyl alcohol, and could be separated by dialysis into two fractions, one of which contained 2.97% of hydrochloric acid and was almost insoluble in methyl alcohol, while the other contained 11.13% of hydrochloric acid.[1]

In order to prepare the free gelatones from their hydrochlorides, a slight excess of silver sulphate should be added to the solution, the excess of silver removed from the filtrate by hydrogen sulphide, and the sulphuric acid by an equivalent amount of baryta water. The gelatones thus obtained are soluble in all proportions in water, but are insoluble in alcohol or ether. Their aqueous solutions are acid to litmus, but do not turn Congo-red to blue. The percentage of carbon in gelatones is somewhat less, and that of hydrogen rather higher, than in gelatin, which fact is in agreement with the view that they are products of the hydrolysis of the latter substance. Paal considers that the gelatin molecule is resolved by hydrolysis into gelatones of gradually decreasing molecular weight, till a point is reached at which they are split up with formation of amino-acids, lysine, lysatine, etc., (page 129); and that, as the molecule of gelatin, like that of albumin, consists of two protein atom-complexes which suffer hydrolysis with different degrees of facility (the salts containing a high proportion of acid being the more readily converted into amino-acids and other non-protein products), the simpler products of decomposition are always mixed with unaltered gelatones.

In a later paper (*Ber.*, **31**, 956), Paal states that the hydrochlorides containing the lower proportions of acid are precipitated by saturating their solutions with ammonium sulphate, and hence are salts

[1] From determinations by the cryoscopic method in aqueous solution, and by the boiling-point method on solutions of gelatone hydrochlorides in water, methyl alcohol, and ethyl alcohol, Paal concludes that the molecule is less as the percentage of acid increases, the molecular weight of the free gelatones being about 300 in 3 cases, and 215 in a fourth; while gelatin itself has a molecular weight of about 900. (Hofmeister's formula for gelatin, $C_{102}H_{151}O_{39}N_{31}$, corresponds to a molecular weight of 2433.) Paal's results further show that the gelatone hydrochlorides are stable in solution in ethyl alcohol, but that when in solution in water or methyl alcohol they are dissociated into equal molecules of gelatone and hydrochloric acid.

of *gelatoses*, analogous to the proteoses (page 147). The portions not precipitated by ammonium sulphate consist of salts of gelatin-peptone or *gelatone*. On the average, these latter salts contain, when anhydrous: ash, 0.18 to 0.58%; hydrochloric acid, 10.38 to 13.14; C, 43.28 to 45.95; and H, 6.43 to 7.13%. By dialysis, Paal effected a further fractionation of these salts. Thus from the diffusate of a hydrochloride which was hardly soluble in ethylic alcohol, but which dissolved readily in cold methyl alcohol, Paal obtained a yield of more than 60% of the original substance, containing: ash, 0.89; HCl, 13.44; and carbon and hydrogen corresponding to C, 48.86, and H, 7.31% in the free gelatone. On dialysing this last product into water, a further separation was effected, the portion diffusing during the first 24 hours being an extremely hygroscopic yellow mass, which was very soluble in alcohol, contained 16.06% of hydrochloric acid, and yielded 1.21% of ash on ignition. The portion which diffused during the second period of 24 hours was much less soluble in alcohol, less hygroscopic, and contained only 9.38% of hydrochloric acid.

Paal finds these salts to be partially precipitated by phosphotungstic acid, with liberation of the gelatone, whilst the unprecipitated portion gives a distinct biuret reaction.[1] By treating the phosphotungstic precipitate with barium hydroxide in excess, a *barium gelatone* was obtained; and this reacted with ferrous sulphate, yielding an aqueous solution of *ferro-gelatone*. This latter substance absorbed atmospheric oxygen, especially on warming, forming free gelatone, which remained in solution, and ferric hydroxide, which was quantitatively precipitated.

Paal finds that when gelatone salts are dissolved in absolute alcohol and the solution saturated with hydrochloric acid gas, while warmed on the water-bath under a reflux condenser, merely a trifling increase occurs in the amount of combined hydrochloric acid, further peptonisation only taking place in presence of water.[2]

Chittenden and Solley (*J. Physiol.*, **12**, 23; abst. *J. Chem. Soc.*, 1891, 949) have investigated the products of the digestion of gelatin by the gastric and pancreatic enzymes. They obtained three distinct products, two of which are related to the albumoses, and the

[1] Paal attributes this result to the formation of products intermediate in nature between peptones and amino-acids.

[2] C. Paal has continued his researches on the *salts of gelatone* (*Ber.*, **31**, 956; abst. *J. Chem. Soc.*, 1898, 1, 456).

third to Kühne's albumin-peptone. The two former substances, *protogelatose* and *deuterogelatose*, are formed both in gastric and in pancreatic digestion, and are distinguished from the third product, which is a true gelatin-peptone or *gelatone*, by being (like albumoses) precipitated on saturating the liquid with ammonium sulphate. Protogelatose is partially precipitated by saturating its neutral solution with sodium chloride, and is completely precipitated on subsequently adding a little acetic acid. Protogelatose yields a heavy precipitate with hydrogen platinichloride. Deuterogelatose is not precipitated by either of these reagents. By further ferment action, protogelatose is converted into deuterogelatose, and finally into gelatin-peptone or gelatone. No trace of heterogelatose was obtained. Chittenden and Solley give the following analytical figures representing the percentage composition of the gelatin employed, and of the gelatoses obtained:

	Gelatin used	Products of gastric digestion		Products of pancreatic digestion	
		Proto-gelatose	Deutero-gelatose	Proto-gelatose	Deutero-gelatose
Carbon	49.38	49.98	49.23	49.45	49.07
Hydrogen	6.81	6.78	6.84	6.61	6.66
Nitrogen	17.97	17.86	17.40	17.81	17.52
Sulphur	0.71	0.52	0.51	0.57	0.65
Oxygen	25.13	24.86	26.02	25.56	26.10
	100.00	100.00	100.00	100.00	100.00
Ash	1.26	1.98	1.08	1.75	1.08

P. A. Levene (*Z. Chem.*, 1902, **37**, 83) obtained, by digesting gelatin with papain, gelatoses whose composition was substantially the same as those given above. Levene further found the gelatoses contained more glycine (amino-acetic acid) than the original gelatin, and, what was still more surprising (*ibid.*, 1904, **41**, 8), more than the gelatin-peptone. This was, however, due to the fact that the glycine remains in solution upon the precipitation of the peptone by phosphotungstic acid.

Bacterial Decomposition of Gelatin

The products of the decomposition of gelatin under the action of bacteria have been studied by Rideal and Stewart (*Analyst*, 1897, **22**, 255). The following is a tabulated summary of the results obtained, the figures being grm. per 100 c.c. of the liquid:[1]

	Total nitrogen	Gelatin and albumoses	Albumoses	Ammonia and volatile bases	Bases and extractives	Peptone N. unaccounted for	Gelatin	Albumin and cellulose
Original gelatin......	1.242	1.109	0.514	0.003	0.061	0.069	0.595	... .
Original gelatin......	1.246	....	0.528	0.004	.. ....	....	0.581	... ...
After incubation at 20 to 21° with—								
B. prodigiosus, 14 days.....	1.232	1.024	0.409	0.049	....	....	0.615	. ..
B. fluor. liquefaciens, 1 day............	1.33	1.141	0.402	0.028	....	....	0.739	. ....
B. fluor. liquefaciens, 2 day	1.256	0.682	0.297	0.045	0.105	0.424	0.385	......
B. fluor. liquefaciens, 3½ days..........	1.267	0.242	0.135	0.063	0.486	0.476	0.107	....
B. fluor. liquefaciens, 16 days...........	1.26	0.216	0.081	0.168	0.742	0.124	0.135	0.01

It will be noticed that the original dry gelatin contained roughly 50% of matter precipitable by Stutzer's reagent (so-called albumoses). In the early stages these albumoses seem to have been first attacked by the organism; but subsequently their decomposition proceeded at an equal rate with that of the true gelatin.

A notable feature is the small production of ammonia and volatile bases, in view of the fact that substances such as trimethylamine, indole, and skatole have been generally stated in analyses by other observers to be constant accompaniments of the liquefaction of proteins.[2]

The figures for the nitrogen unaccounted for, which would be almost entirely peptones, are interesting. The original gelatin solution contained 0.069 grm. per 100 c.c., but this gradually

[1] The gelatin and albumoses were determined by precipitating the solution with saturated ammonium sulphate solution, dissolving the precipitate in warm water, and dividing. The total nitrogen was determined in one-half of the solution, and in the other, very exactly, the SO_3, the nitrogen due to the corresponding ammonium sulphate being deducted from the total ammonium sulphate.

The albumoses were determined separately by precipitating a portion of the original solution with Stutzer's reagent, washing, and estimating the nitrogen in the precipitate.

The volatile bases were determined in a portion of the original liquid by distillation with caustic soda and titration of the distillate. A further portion of the original was precipitated with alcohol, filtered, and the nitrogen-content of the filtrate regarded as existing as bases and extractives.

[2] That this was not due to the volatilisation of such bodies during the incubations was proved by the comparative absence of odour and of strong alkaline reaction in the air of the flasks, and in such loss not being indicated by the total nitrogen. Previous investigations by Rideal and Stewart with proteins showed that the quantity of ammonia produced was insignificant, amounting, even after 16 days' incubation with *B. fluorescens liquefaciens*, to only 0.168 grm. of nitrogen per 100 c.c., corresponding to 0.204 grm. of ammonia.

increased as the liquefaction proceeded, until, with *B. fluorescens liquefaciens*, it reached the maximum figure of 0.476, when the medium was just entirely liquefied, and then diminished, after 16 days, to 0.124, showing that the bacillus, after bringing about the decomposition of nearly all the gelatin and albumoses, subsequently attacked the peptone, which had meanwhile been produced, with a corresponding increase of bases and extractives soluble in alcohol.

It is known that certain bacteria produce peptonising enzymes, and in the case of the *B. fluorescens liquefaciens* the organism appears, as shown above, to have subsequently fed on the peptone produced by its enzyme.

Formogelatin

On adding a solution of formaldehyde—such as commercial formalin—to an aqueous solution of gelatin, no change occurs unless the gelatin solution be very concentrated or contains free alkali, but on evaporating the liquid to dryness, the gelatin is converted into an insoluble substance known as formogelatin. To effect complete conversion of the gelatin into this substance, any free acid should be previously neutralised by agitation with precipitated calcium carbonate or other means. On treating the residue left on evaporation with boiling water any trioxymethylene formed from the formaldehyde is dissolved, while the formogelatin is unaffected.

From the small proportion of formaldehyde required to produce formogelatin, it is very doubtful whether that substance is a definite compound.[1] Acrylic aldehyde is stated to yield a similar product, but acetic aldehyde only reacts in the absence of water.

Formogelatin is quite insoluble in either cold or boiling water, but is completely dissolved by treatment with diluted sulphuric acid (1.34 sp. gr.) for 12 hours, whereas the corresponding compound from casein (page 185) remains undissolved. Formogelatin swells on soaking in water, but much less than the gelatin before treatment.

R. H. Bogue (*Chem. Met. Eng.*, 1920, **23**, 7–9) has investigated the effects of varying percentages of formaldehyde on gelatin.

[1] This mooted question is discussed *e. g.* by D. Jordan-Lloyd, "Chemistry of the Proteins" (J. & A. Churchill, London, 1926) p. 258. That stoichiometric combination results under certain conditions is obvious, because Sörensen's method of estimating amino-groups is based on it.

$$\underset{\displaystyle COOH}{R} - NH_2 + H.CHO \rightarrow \underset{\displaystyle COOH}{R} - N{:}CH_2 + H_2O.$$

On adding 10% formaldehyde solution to 5.5 to 22% gelatin solution, Bogue found: (1) viscosity varied directly with the amount of formaldehyde added; (2) jelly strength varied inversely with the amount added; (3) the viscosity increases with time; (4) viscosity decreases with rise in temperature up to about 40°, above which it rapidly increases to the setting point.

According to E. O. Beckmann (*Chem. Centr.*, 1896, **2**, 930) the same insoluble substance is obtained by the action of formaldehyde on gelatin which has lost its power of gelatinising by prolonged heating,[1] but he states that gelatin-peptone is not similarly acted on. "Albumin-peptone" and "tryptone" are also said to be unaffected. Beckmann based on these facts a method for the determination of gelatin in meat-extracts.

According to A. Zimmermann, methylene blue colours formogelatin but not ordinary gelatin.

Formalin-gelatin has received several practical applications. Schröder prepares it (abst. *Pharm. Jour.*, 1896, **2**, 63) by adding 2% of commercial "formalin" (40% aqueous solution of formaldehyde) to a warm solution of gelatin in its own weight of water. The liquid is stirred, the resulting mass covered with "formalin," and allowed to stand for some time. The product is then powdered, well washed with water, and dried. H. K. van Vloten (*J. Soc. Chem. Ind.*, 1896, page 553), who prepared the compound in a somewhat similar manner, states that powdered formogelatin can be introduced into wounds without producing irritation.

The following method has been proposed by G. Romijn (abst. *J. Soc. Chem. Ind.*, 1896, **15**, 679) for the detection of unaltered gelatin in formogelatin. About 0.5 grm. of the sample is mixed with 10 c.c. of water and heated for 10 minutes in the water-bath with frequent shaking. The mass swells considerably, but particularly so in the presence of unaltered gelatin, which goes into solution. If the liquid is now passed while hot through a dry filter, it will gelatinise on cooling if sufficient unaltered gelatin be present. The separation of the gelatin may be accelerated by placing the filtrate in a freezing mixture and allowing it to melt slowly.

[1] To prepare an insoluble film of formogelatin from altered gelatin, A. Zimmermann (*Eng. Patent*, 1894, No. 23585) dissolves 75 grm. of gelatin in 500 c.c. of water, and boils the liquid for 2 days, replacing the water as it evaporates. He then adds 4.2 grm. of commercial formalin, containing 40% of formaldehyde. The liquid remains clear for a prolonged time, but on evaporation yields insoluble formogelatin. Gelatin altered by boiling with dilute acids or alkalis acts in the same manner.

0.5 grm. of the sample is mixed with 5 c.c. of water and 1 c.c. of sodium hydroxide solution. The mass swells considerably, but much more strongly in the presence of unaltered gelatin. If now a mixture of 2 c.c. of normal silver nitrate solution and 0.5 c.c. of ammonia be added, the mass should begin to darken within 1 or 2 minutes, and after 5 or 10 minutes the liquid should have acquired a violet-brown colour. If much free gelatin is present, the colour makes its appearance more slowly, and assumes a pure brown shade, without any violet. Pure gelatin does not produce any coloration until after the lapse of a few hours.

Glue and Gelatin[1]

It is not easy to draw a sharp line of demarcation between glue and gelatin. Broadly speaking, glue is impure, dark coloured, or low-test gelatin, and usually contains considerable quantities of "chondrin," gelatoses, and gelatones. Gelatin is distinguished from glue by its purity, light colour, clearness, and high jelly strength.[2] The same raw material may yield glue or gelatin, according to the skill and care bestowed upon its manufacture.[3] A sharp distinction should be made between *technical gelatins* intended for use in manufacture and the arts, and *food gelatin* intended for alimentary purposes. (See page 174.)

In the manufacture of glue and gelatin, the raw material or "stock," after being cleaned and "plumped" by appropriate treatment (see below), is subjected to the action of hot water or steam. The resulting liquor[4] or "soup" is skimmed to remove fatty matters, bleached, clarified, filtered and evaporated if desired, and then run into galvanised pans, or on water-cooled slabs, where it sets to a jelly. The jelly is cut into sheets, which are spread on nets of galvanised wire or fish cord, and dried in a current of warm dry air circulated by fans. Rotary drums, automatic chilling, cutting and spreading machines (Kind's patent) and spray-drying devices are in increasing development and use.

[1] An extensive bibliography of glue and gelatin, including more than 150 references, was given by Dr. Rudolph Ditmar, in the *Z. Chem. und Ind. der Kolloide*, 1906, 1, 80.

[2] The French recognise a class of gelatin-glues (*colles gelatines*) intermediate between gelatin and glue.

[3] Stout consommé, which sets to a jelly when cold, although a food product, would yield on drying only a low-grade glue.

[4] The liquor thus obtained is known as a "run" or "boiling." Since prolonged exposure to heat causes hydrolysis, and injures the quality, it is customary to make a number of successive runs or boilings, which may be combined or kept separate as desired. As a rule, first runs are best both in colour and strength, second runs next, and so on. (See H. J. Watson, *J. Soc. Chem. Ind.*, 1904, 23, 1189.)

The chief sources of gelatin and glue are bones, sinews, and skins or hides.[1] It is frequently but erroneously stated[2] that glue is made from hoofs and horns, but these consist of keratin and yield no glue. The bony support of the horn, or horn-pith, yields a particularly fine gelatin; so also do calf and pig skins.

Most hide stock consists of trimmings, skivings and fleshings from tanneries; besides these, commercial hide stocks include rawhide cuttings, rabbit skin shreds from makers of hatters' felt, calves' pates, and occasionally worn out bits of rawhide, such as loom-pickers and Turkish moccasins. Various parts from the calf, cattle, goat, sheep, rabbit, pig, and horse each yield characteristic glues, and are valued about in the order given.

The treatment of the stock depends upon the technique of the particular factory, and the use for which the finished glue is intended. Bones may be boiled without even washing, but usually the grease is first steamed out, or else removed by volatile solvents. The clearest and best bone glues are obtained by leaching the bones with dilute acid,[3] which dissolves out the lime salts and leaves the collagenous matter. Such crushed leached bone enters commerce under the name of *osseine*, and is largely used for making gelatin. Hide stock, sinews, and leached bone are soaked in dilute lime water until sufficiently swollen or "plump," and after washing, the last traces of alkali are neutralised with acid. Sulphurous acid is frequently used as a bleach, the remaining traces being oxidised by hydrogen peroxide.

Glue and gelatin occur in commerce in a great variety of forms (sheet, flake, shred, ground, powdered, etc.). While gelatin is usually colourless or else clear pale-yellow or brown, glue varies in colour from milk-white to almost black, and all possible shades of brown and yellow, transparent or opaque.

Although appearance[4] is no certain criterion of quality, glue is often erroneously judged by its colour, clearness, fracture, etc.

[1] Glues made from bones are called bone glues. That made from hide should be called hide or skin glue, though it is sometimes called *leather glue*, which is a misnomer, because leather yields no glue. Patents have however been taken out for de-tanning leather and making glue from the recovered hide. Besides the two classes referred to, the Germans recognise a class of mixed glue (Misch-leim), made from a mixture of bone and hide stock.

[2] The Oxford Dictionary and the Standard Dictionary contained this error.

[3] Hydrochloric acid is most generally used. In the Grillo and Schroeder process (*Eng. Pat.* 2175 (1894). *J. Soc. Chem. Ind.*, 1894, **13**, 408), sulphur dioxide is passed over the bones, which absorb 11–12 % with the formation of insoluble $CaHPO_4$ and $CaSO_3$. Bones so treated are readily disintegrated by water at 100°, the gelatin passing into solution.

[4] Glue in small oblong cakes (3 in. × 6 in.) is known as *Cologne glue*. *Scotch glue* is dark reddish-brown, in large oblong sheets. *French glue* is generally about 9 in. square, and is frequently stamped with a trade-mark (*medal glue*). Glue made opaque with oxide of zinc or lithopone is often called *Russian glue*, although in America thin-cut opaques are commonly known as white-shell glues. *Ribbon glue* comes in thin strips about 2 in. × 6 in., and *noodle glues* in thicker and narrower pieces. The dark, heavy noodle glue, popular in Eastern countries, is called *Bazaar glue*. The form in which a glue is dried or cut, does not, of course, affect its quality.

Since glue is used for a great variety of widely differing purposes, the *use for which the glue is intended* should always be borne in mind when subjecting it to test or technical examination. *A number of tests*[1] will, therefore, be given, *which may be conveniently run in consecutive series*,[2] and their practical significance will be pointed out in a few instances.

Thin blown glasses, about 3½ in. high and 2⅜ in. in diameter, are convenient for making tests.[3] 25 grm. of each glue to be tested is broken into small pieces and soaked in 100 c.c. of cold water until thoroughly softened. Thick sheet or flake glues must be soaked over night and should be allowed to stand in a cool place. With the glues under examination, there are at the same time soaked up a number of glues of known strength (standards) for tests of glue should always be comparative. It is desirable and convenient to use the standards hereafter described. (See page 163). With high test glues, or in cold weather, less glue may be used, provided that the unknown glues and the standards are treated exactly alike. In warm weather low test glues must sometimes be tested 30 to 100. Gelatins are usually tested 10 to 100, or even 3 to 100.

When the glues are thoroughly softened, the glasses are immersed in a water-bath, and their temperature raised to 80° with constant stirring to insure complete solution.[4] Many prefer to test glue at a lower temperature to avoid hydrolysis, evaporation etc. In using other viscometers and jellometers to those here described, the conditions may be varied, *provided that unknowns and standard glues are all treated alike.* Testing with standard glues as a check is much safer for the general analyst than "absolute" determinations which, because of the potent influence of temperature and other factors, might lead to considerable discrepancies. With thick-cut glues, care must be taken to see that no undissolved pieces stick to the bottom of the glass. The following tests are then made in the order given.

[1] E. G. Clayton (*J. Soc. Chem. Ind.*, 1902, **21**, 670), at the conclusion of a paper on the technical examination of glue, says "while it would be rash to form a judgment on glue from a single test, the evidence afforded by a number may be irresistible. The expert's wisest system appears to be, not to rely upon single short-cut tests of general quality, but to employ a number of methods, including any having especial bearing on the prospective or present uses of the glue, and then base his conclusions on a consideration of all the results together."

[2] See J. Alexander, *J. Soc. Chem. Ind.*, 1906, **25**, 158. A typical test sheet, showing results of this series of tests, is given on page 158.

[3] The National Association of Glue Manufacturers (U. S. A.) use a wide-mouthed bottle with a rubber stopper. They recommend taking the viscosity at 60° C., and exact details of the tests they recommend, are given in *Ind. Eng. Chem.* for 1924, **16**, 310. There was recently published a revision, brought up Nov. 29th, 1929. An epitome of these tests is given on p. 165, following the comparative table of different glue grades.

[4] As hydrolysis occurs at this temperature, the heating should not be prolonged. Standard glues must be kept in metal or glass so as to avoid change in moisture content.

No. 1496
Dec. 15th.

LABORATORY TEST SHEET[1]

(20 Grm. Glue to 100 c.c. Water. Soaked 16 Hours)

Marks	Visc.	Swt.	Grease	Foam	Alk.	Acid	C. Set.	Sngth.	Jelly	Reaction
1. Low-grade hide glue	17¾	1	2	1	2½	1	14	58	Separates.	This column used to receive litmus-paper strips
2. Medium-grade hide glue	19¾	1	3	1	2	1	10	82	Opaque.	
3. Medium-grade hide glue	20¼	1	2	1	1	1	5	102	This column used to describe the jellies when desired	
4. High-grade hide glue	24	1	1½	1	1	1	3	140		
5. High-grade hide glue	27	1	1	1	1	1	1	160		
6. Low-grade bone glue	16½	1	1	3	1	1½	18	34		
7. Medium-grade bone glue	17	1	1½	1	1	2	11	76		
8. Medium-grade bone glue	18½	1	2	1	1	1	8	90		
9. High-grade bone glue	17¾	1	1	1	1	2	4	110		
10. Second run Osseine stock	21	1	1	1	1	1	2	150		
11. Third run Osseine stock	18¾	1	1	1	1	1	6	104		
12. German oblong *sheet* (*bone*)	16	1	1	2½	1	1	16	48		
13. English oblong sheet (bone)	16½	1	1	1½	1	1	12	60		
14. Dutch oblong sheet (bone)	16¾	1	1	2	1	1½	13	56		
15. French square sheet (bone)	16	1	1	2	1	1½	17	50		
16. Italian square sheet (bone)	17	1	1	1½	1	2	15	52		
17. German "*Lederleim*"	18¾	1	2	1	1	1	9	80		
18. English "skin glue"	18½	1	1½	1	1½	1	7	90		

[1] For the sake of brevity, figures are adopted to describe the various characteristics:
1 = perfect or free; 2 = slight; 3 = considerable; 4 = much; 5 = very much.
With each test must be run a sufficient number of standard glues to permit the proper grading or interpolation of the unknown glues.

Reaction.—This is determined with strips of litmus paper.

Where the degree of acidity or alkalinity is desired, a separate titration is made, or the pH is determined.

Viscosity or Running Test.[1]—The viscosity is taken by running the hot glue solution at 80° from a pipette, and noting the time of efflux by a stop-watch. The relative viscosities are thus fixed in seconds.

The pipette adopted as standard has the following dimensions:

Capacity	45 c.c. of water at 80°
Internal diameter of effluent tube	6 mm.
External diameter of effluent tube	9 mm.
Length over all of effluent tube	7 cm.
Smallest diameter of outlet (about)	1.5 mm.
Outside diameter of bulb	3 cm.
Length of bulb	9.5 cm.
Length of upper tube	22 cm.

This pipette should permit the efflux of 45 c.c. of hot water at 80° from the glue bath in exactly 15 seconds. The viscosities of glues vary widely, as may be seen from the table of standards on page 164.

Great care must be taken to make 2 pipettes that will give concordant results. The size and shape of the outlet hole, and the length and diameter of the effluent tube are the chief factors controlling the time of delivery. The efflux hole is made by cutting the effluent tube square across, and holding it vertically in a Bunsen flame with constant rotation. As the glass softens, the hole gradually draws together, and after a few trials can be brought to the desired size. It is desirable to have the lower graduation point just where the effluent tube joins the bulb, for otherwise in glues of high viscosity there is much uncertainty caused by dribbling of the last few drops.

While running the pipette may be kept in a simple thermostat, consisting of a water-bath specially made for the purpose. The projecting effluent tube is protected by a mica cylinder through which the end-point is observed. When the thermostat is used a small piece of rubber tubing controlled by a pinch cock is slipped over the upper end of the pipette, or a glass stopcock may be fused on.

After each determination, the pipette is washed out with hot water from the glue bath, and care must be taken that no undissolved glue, glue "skin," slime, or other obstruction clogs the outlet, even momentarily, during use.

[1] This is sometimes known as Fels' test. As a rule, bone glues show lower viscosity than hide glues of equal jelly strength.

More complicated viscometers, as Engler's[1] (see *J. Soc. Chem. Ind.*, 1890, **9**, 654) or the Rideal-Slotte (see *J. Soc. Chem. Ind.*, 1891, **10**, 615) may be used, but they are cumbersome and slow of operation, and therefore impractical in routine work, where many determinations are made.

Odour.—To an experienced nose, the odour of the hot glue solution usually gives some indication of the raw material from which it is made. Decomposition is readily detected, although it is frequently masked by antiseptics or essential oils. Glues are rated as "sweet" or "off" as the case may be. With food gelatins, freedom from odour is highly desirable.

Grease.—A brush-full of the glue solution is mixed with a little aniline or other colour, and painted out on a piece of white paper, when spots or "eyes" appear roughly proportionate to the amount of grease present. The grease is recorded arbitrarily on a comparative basis. For an exact estimation of fat in glue, etc., Kissling[2] dissolves 20 grm. of the sample in 150 c.c. of water containing 10 c.c. of hydrochloric acid of 1.19 sp. gr. The liquid is heated for 3 or 4 hours on a water-bath under a reflux-condenser. The solution is cooled, 50 c.c. of petroleum-ether added, the liquid well shaken, and, after standing until clear, a known measure of the solvent is drawn off, evaporated, and the residue weighed.

Foam.—Foam is determined by agitating the glue solution with a rod or mechanical agitator, and like grease, is recorded comparatively as to its amount and permanency. Some of the conditions affecting the foaming of glues are discussed by Trotman and Hackford (*J. Soc. Chem. Ind.*, 1906, **25**, 104) and H. J. Watson (*ibid.*, page 209).

Comparative Set.—The glasses are taken from the water-bath, and note is made of the comparative set or order in which the solutions gelatinise. In warm weather, especially with glues of low jelly strength, the glasses must be allowed to stand in ice water. As a rule, glues and gelatins gelatinise in order of their jelly strength and viscosity.

Jelly Strength or "Finger" Test.[3]—When the glue solutions have gelatinised, the glasses are arranged in order of the strength or resiliency of the jellies, as determined by pressure with the finger tips.

[1] J. Fels (*Chem. Ztg.*, 1897, **21**, 56 and 70; *J. Soc. Chem. Ind.*, 1897, **16**, 264) proposed to take the viscosity of a 15 % solution at 30° with Engler's viscometer; later (*J. Soc. Chem. Ind.*, 1901, **20**, 139) he advised using 35°. In practical use, however, glue solutions are usually much warmer and more concentrated.

[2] R. Kissling, *Chem. Ztg.*, **11**, 691, 719, abst. in *J. Soc. Chem. Ind.*, 1887, **6**, 365.

[3] This is sometimes known as Kissling's test.

Notwithstanding the personal equation, expert operators may quickly obtain much more uniform results than are given by the various mechanical devices, unless meticulous and time-consuming precautions are observed.

The unknown glues naturally group themselves as stronger or weaker than the several standards, and if the proper selection of standards has been made, the unknown glues can, if necessary, be graded in between the standards (see table on page 158).

Lipowitz (*Neue Chem. tech. Unters.*, Berlin, 1861, page 37) first proposed the relative consistency of the jelly and its capacity for bearing a weight, as a test for the value of a glue. J. Fels (*Chem. Ztg.*, 1897, 56 and 70) described this as "correct as a comparative method."

The value of Lipowitz's method as a practical test of the quality of glue has also been confirmed by Heinze. The instrument used is made as follows: A saucer-shaped piece of tinned iron, 1 in. in diameter, is soldered in its concave side to a stout iron wire having a small tin funnel fixed to the other end. The arrangement is placed vertically in the beaker so that the saucer rests on the jelly, and the whole is supported by a slip of metal placed across the top of the beaker. By placing *shot* in the funnel a gradual increase of weight on the jelly is produced, until the saucer breaks through. The weight it is found necessary to use to rupture the jelly is a measure of the tenacity of the glue.

Instruments of this type are inaccurate for reasons that will be given below. They have, however, been largely used, and since shot has generally served for the added weight, the test has been known as the "*shot test*." One modification of the "shot test" is based upon the time required for rods of different weight to sink a certain distance in the jelly. Another modification, perhaps the best, consists in determining the weight required to produce a certain compression of the jelly in the glass, without breaking it. In this modification, the upright rod in moving, actuates a rotary pointer, and when this reaches a certain point on its scale, the weight resting on the glue indicates the jelly strength; or the motion produced by a definite weight is recorded.

On a somewhat similar principle to this last modification is the apparatus devised by E. S. Smith (U. S. Pat. 911277; see *J. Soc. Chem. Ind.*, 1909, **28**, 252) for measuring jelly strength. It consists

of a pressure chamber one side of which is closed with a thin elastic rubber diaphragm, a rubber bulb to produce pressure, and a manometer to measure the pressure produced. Upon compression of the bulb, the diaphragm displaces the jelly, and the pressure required to produce a certain displacement is taken as the jelly strength.

All forms of apparatus which depend upon the breaking or compression of the jelly in glasses, are subject to two sources of error. The glasses usually vary in diameter, thus forming a surface of variable area, and there is always formed a skin of greater or less thickness, which interferes with the accuracy of the test. To avoid these difficulties, J. Alexander (see U. S. pat. 882731; *J. Soc. Chem. Ind.*, 1908, **27**, 459) devised a jelly tester to determine the resiliency of jelly blocks free from containing walls, the skin being placed at the bottom. It consists of a brass cylindrical vessel supported like a gas tank by four vertical rods, against which it slides with almost frictionless roller bearings. This brass cup is allowed to rest on a truncated cone of jelly of definite size and composition, and at a definite temperature; and shot are gradually poured into the cup until a definite compression of the jelly is observed. Beneath the cup are two vertical adjustable brass uprights 3.5 cm. high, connected with an electric bell circuit; so that when the cup reaches their level, it completes the circuit and the bell rings.

The weight of the brass cup, plus the weight of the added shot, gives a figure that expresses the jelly strength.

The jellies are cast in round brass cups 6 cm. high, 5.5 cm. in diameter at the open top, and 5 cm. at the bottom, which is closed with a tight-fitting external friction cap. If the jellies do not push out readily on removing the cap, the covered cup may be dipped for an instant in hot water, taking care that no material loss results. The liberated truncated cones should be exactly 4.5 cm. high, the cups being filled only to that level. They are placed in a thermostat until they reach the desired temperature.

This instrument was used in fixing the following standards, which cover the range of jelly strengths found in commercial glues and gelatins. Many other types of gelometers are described in the literature, or have been privately devised and used. Sheppard, Sweet and Scott (*J. Ind. Eng. Chem.*, 1920, **12**, 1007) describe a torsion dynamometer. Sheppard and Sweet (*J. Ind. Eng. Chem.*, 1923, **15**, 571) describe a plunger type of instrument, and (*ibid.*,

1924, **16**, 593) discuss some unsolved factors in jelly strength determination. See also Sheppard, Sweet and Benedict (*J. Amer. Chem. Soc.*, 1922, **44**, 1857) on the effect of *p*H on the elasticity of gelatin jellies.

Standards.—The selection of standards is of great importance, for once they are taken, all unknown glues are measured by them. Most published results of glue tests cannot be compared with each other, because of the great diversity of methods and glues used, and the absence of standards. That definite standards of jelly strength will simplify and harmonize the grading of glue is self-evident.

Sixteen arbitrarily established, nearly equidistant *grades*, cover the usual range of jelly strengths. The grades are given values from 10 to 160, thus allowing 10 points between each grade. The following table gives the viscosities (as determined by the standard pipette, see page 159), and the jelly strengths (as determined by Alexander's jelly tester, see above) of these 16 standards. The determinations were made on solutions of the glues containing 25 grm. of glue to 100 c.c. of water. For the standard viscosities there are taken the average of laboratory tests extending over many years, and inasmuch as standards of the same jelly strength are apt to vary in viscosity, there is indicated in the table the reasonable limit of such variation. Glues often greatly exceed these limits, but should not be chosen as standards.

The higher standards, from 60 and upwards, should be neutral hide glues, clear, clean, well made, free from any odour of decomposition, and practically free from foam and grease. For the lower standards, bone glues should be chosen, because most low-test glues are bone glues, and, besides, low-test hide glues usually have abnormally high viscosities.

Keeping Properties.—After determining the jelly strength, the the glasses are allowed to stand uncovered for several days at room temperature to observe the relative keeping properties of the jellies. If it is desired to know the keeping properties under special conditions, these conditions must be simulated.

After observing the keeping properties, the glasses are washed out, and the routine tests referred to on page 165 *et seq.* There will be given below a number of additional tests which are often referred to, with comments upon their value.

Many American manufacturers and dealers use for comparative testing the so-called "Cooper grades" which were established many years ago by Peter Cooper, a pioneer American manufacturer, as grades under which his glues were sold.

In all probability, these were originally based upon the distance a certain weighted rule would compress a certain dish or bowl of jelly. The "No. 2" allowed it to sink 2 in., the "No. 1," only 1 in., while the intermediate grades were measured in eighths of an inch.

Besides the Cooper grades given, for comparison, there are also added the grades proposed by Robert H. Bogue ("Chemistry and Technology of Gelatine and Glue"; also Chem. Met. Eng. for 1920) as well as the grading adopted by the National Association of Glue

COMPARATIVE TABLE OF AMERICAN GRADES OF GLUE AND GELATIN

Alexander				Bogue grades	Natl. Assn. Glue Mfrs. (U. S. A.)		
Standard grades	V.[1]	J. S.[2]	Cooper grades		Grade	V.[3]	J. S.[4]
10	15.5 ± 0.25	......	....	......	1	20	10
20	16 ± 0.25	......	....	1	2	24	27
30	16.5 ± 0.25	......	2	2	3	28	47
40	17 ± 0.25	1,701	1 7/8	3	4	32	70
50	18 ± 0.5	2,324	1 3/4	4	5	37	95
60	19 ± 0.5	2,948	1 5/8	5	6	42	122
70	20 ± 0.5	3,572	1 1/2	6	7	47	150
80	21 ± 0 5	4,196	1 3/8	7	8	53	178
90	22 ± 0.75	4,820	1 1/4	8	9	60	207
100	23 ± 0.75	5,443	IX	9	10	67	237
110	24 ± 0.75	6,067	1	10	11	75	267
120	25 ± 1	6,691	1 extra	11	12	83	299
130	26 ± 3	7,314	A extra	12	13	92	331
140	28 ± 5	......	....	......	14	102	363
150	34 ± 8	......	....	......	15	113	395
160	40 ± 12	......	....	......	16	125	428
					17	138	461
					18	152	495
					19	167	530
					20	183	565
					21	200	600

1 V. = viscosities in seconds at 80°, solution 25 − 100.
2 J. S. = jelly strength in grams at 10°, Alexander jelly tester.
3 V. = viscosity in millipoises, lower limit; special calibrated pipette.
4 J. S. = lower limit of jelly strength in grams, Bloom gelometer.

Manufacturers[1] (American) Oct. 10th, 1923 (see *Ind. Eng. Chem.*, 1924, **16**, 310). See also *International Critical Tables*, Vol. II, p. 218.

The National Assn. of Glue Manufacturers (U. S. A.) use 12.5% solutions (15 ± 0.05 grms. commercially dry glue to 105 ± 0.25 c.c. of distilled water, soaked until thoroughly softened, in an ice box at 10–15°). Viscosities are taken with a calibrated jacketed pipette at 60° ± 0.2°. The solutions are handled in wide mouth bottles of 150 c.c. capacity, 59 mm. internal diameter, 85 mm. overall height, closed with a No. 9 rubber stopper whenever feasible, to avoid evaporation. The jelly strength is taken with the Bloom gelometer, which automatically measures the quantity of shot needed to produce a fixed depression of the carefully chilled jelly by a downward acting plunger. For full details, and a critical discussion of the numerous factors which may influence the result, reference must be made to the original paper in Industrial and Engineering Chemistry, 1923, and to the revised directions published from time to time (last in 1930).

Water.—From 2 to 3 grm. of glue are roughly granulated and dried at 110 to 115° until constant in weight. The estimation of water is without much practical significance if the glue is commercially *dry;* for the more moisture a glue contains, the weaker it will show on the viscosity and jelly tests.

Ash.—The ash of glue varies considerably, both in amount and composition. In the case of some bone glues the ash consists largely of phosphates, whilst in hide glues considerable calcium sulphate or other calcium salts (formed upon neutralisation of the lime used in preparing the stock), are apt to be present. Chalk, clay, zinc oxide, and lead carbonate and sulphate (which are sometimes added as whiteners) and zinc sulphate, which is used as a preservative, are often found. Titanium oxide is also being used as a whitener, since this material is now obtainable at a reasonable cost.

For the estimation of ash, a weighed quantity (2 or 3 grm.) of glue is incinerated in a large covered platinum crucible, a few drops of nitric acid being used if necessary, to ensure the oxidation of the carbon. The heating must be conducted with care, as the glue at first intumesces violently. The ash of bone glue is supposed[2] to be fused by the heat of the Bunsen burner, to be neutral, and to

[1] A revision, brought up to Nov. 29th, 1929, was issued by the National Association of Glue Manufacturers, Inc., 55 West 42nd St., N. Y. City.

[2] See Kissling, *Chem. Ztg.*, **11**, 691 and 719; *J. Soc. Chem. Ind.*, 1887, **6**, 565.

contain phosphoric acid and chlorides; whereas the ash from hide glue is supposed to be infusible owing to the presence of lime, to be alkaline, and generally free from phosphoric acid and chlorides.

The composition of the ash, in reality, depends more upon the nature of the process than upon the raw material used in making the glue, and from a practical standpoint its estimation is generally unnecessary, unless to detect some added adulterant or for the purpose of comparison. Hide and bone glues are frequently mixed, both in the liquor and in the finished form, and it is unsafe to attempt to judge the raw material of a glue on the basis of its ash.

Melting Point.—Strictly speaking, a glue jelly has no absolute m. p., for it softens up gradually and shows no sharp line of demarcation between solid and liquid. In general, the m. p. is proportionate to the jelly strength, although glues of low viscosity *appear* to melt more readily than those of heavier body. The m. p. may be ascertained by the simple apparatus described by N. Chercheffisky (*Chem. Ztg.*, 1901, **25**, 413; *J. Soc. Chem. Ind.*, 1901, **25**, 731); this consists of a 250 c.c. beaker filled with refined paraffin oil, into which is hung a wire with a horizontal end upon which are threaded several small blocks of jelly. When these lose their rectangular form upon warming, the m. p. is read on a thermometer which hangs as near them as possible. For exact work, the beaker may be water jacketed in a larger one.[1]

Another apparatus for ascertaining the m. p. is Cambon's "fusiometer" (see *J. Soc. Chem. Ind.*, 1907, **26**, 703). Perhaps the best way is to reheat the jellies in the test-glasses (see page 157) and note comparatively how they melt.

The m. p. test is uncertain, and takes more time than it is worth, for the jelly test is preferable.[2]

Hygrometric Test (*Cadet's Test*).—This is based on the quantity of water absorbed by a glue on exposure to damp air, and is even more unreliable than the succeeding test.

Water Absorption Test (*Schattenmann's Test*).—A known weight of glue is immersed in water at ordinary temperature for 24 hours. In this time, the high-test glues absorb from 10 to 15 times their weight

[1] See also Sheppard and Sweet, *J. Ind. Eng. Chem.*, 1921, **13**, 423, for a discussion of the setting and melting points of gelatins, and for description of some special apparatus.

[2] The determination of the temperature of gelatinisation, as proposed by K. Winkelblech (*Z. angew. Chem.*, 1906, **19**, 1260; *J. Soc. Chem. Ind.*, 1906, **25**, 769), is also uncertain and troublesome to determine.

of water, whereas weaker glues absorb only 3 to 5 parts.[1] Very low-test glues form a slime rather than a jelly.

Although the best glues take up more water than inferior qualities when immersed in the liquid, well-made and well-dried glues are much less hygroscopic than badly made specimens, or than those prepared from inferior materials. The latter are also liable to undergo putrefaction on exposure to damp.

The water absorption is roughly proportional to the jelly strength, but is so inaccurate that it is hardly worth the trouble involved, unless as a rough approximation.

"Chemical Tests."—The exact estimation of acidity or alkalinity (see page 159) by titration might be included under this category. For the estimation of free acid, Kissling (*loc. cit.* on page 160) suspends 30 grm. of glue in 80 c.c. of water and allows it to stand for several hours. The volatile acids are then driven over by a current of steam. As soon as the distillate amounts to 200 c.c., the distillation is arrested and the contents of the receiver titrated with standard alkali. Sometimes the distillate contains sulphurous acid, in which case a known volume of standard alkali should be previously placed in the receiver.

The hydrogen ion concentration (*p*H or active acidity) of a glue or gelatin solution exercises a material influence on its properties. Bogue (*J. Ind. Eng. Chem.*, 1922, **14**, 439; "The Chemistry and Technology of Gelatin and Glue," p. 500) recommends its routine determination. He states (*lib. cit.*): "If the *p*H value is 4.7, the viscosity, swelling, etc. are low, and the product nearly insoluble. On either side of this point the properties increase very considerably, attaining their maximum on the acid side at *p*H 3.5, and on the alkaline side at *p*H 9. The *p*H value indicates, therefore, not only the reaction of the material, and the degree of acidity or alkalinity of the material, but also the proximity of the substance to the points of maximum or minimum properties. The measurement may be made by either electrometric or colorimetric means.[2]

[1] S. Rideal soaks 10 grm. of the coarsely-powdered sample of glue in water at 15° in a weighed beaker for 48 hours. At the end of this time the water is carefully decanted, and the increase of weight ascertained, the character and odour of the jelly being also noted. Good glues give a firm jelly and absorb from 5 to 9 times their weight of water. In other cases, the product is not jelly but a slimy liquid. According to Kissling the amount of water absorbed by glue affords no indication of its cohesive power, and Feis describes it as an uncertain test. If the sample is *finely* ground or *powdered*, this test cannot be made.

H. R. Proctor (*Kolloidchem. Beihefte*, 1911, **2**, 243) found experimentally that the swelling maximum of gelatin is influenced by the volume of the original jelly from which it was dried. The presence of highly ionised acids enormously increase the water absorption of gelatin.

[2] See W. M. Clark, "The Determination of Hydrogen Ions," Baltimore, Md., 1920. Presence of a material amount of SO_2 or H_2O_2 may affect a colorimetric test.

For the estimation of free organic acid, Kalmann (*J. Soc. Chem. Ind.*, 1890, **9**, 113) dissolves 1 grm. of the coarsely-powdered sample of glue in water on the water-bath, tests the solution with blue litmus-paper, and then titrates the liquid with *N*/10 alkali, using phenolphthalein as the indicator. The liquid is then cooled, a little starch solution added, and *N*/10 solution of iodine added until the liquid becomes blue, the amount of iodine required being equivalent to the sulphurous acid of the sample. The blue colour should then be discharged by adding a drop of a solution of sodium hydrogen sulphite, and then again titrated with *N*/10 alkali and phenolphthalein. The volume required indicates the amount of hydriodic acid formed, according to the equation: $M_2SO_3 + I_2 + H_2O = M_2SO_4 + 2HI$. The result should agree with that of the iodine titration, and affords a proof that the organic matter of the glue has not affected the accuracy of that determination.

The exact estimation of sulphurous acid and sulphites in food gelatin is of especial importance in connection with pure food laws, and will be referred to later.

Since the value of glue depends upon its adhesive power, an estimation of the non-gelatinous substances present is of some value. C. Stelling[1] (*Chem. Ztg.*, **20**, 461; abst. *Analyst*, 1896, **21**, 239) esti-

Description	Number of samples	Non-gelatinous substances		
		Maximum	Minimum	Average
		%	%	%
Gelatin (various kinds)	5	4.53	2.53	3.39
Glue	5	4.70	2.00	3.49
Hide glue	3	7.60	4.30	5.73
Bone glue from acid bones	4	11.84	9.24	10.33
Bone glue from acid bones	3	16.78	13.16	15.15
Pressed glues from neutral bone powder	17	32.10	14.30	20.66
Whale glue	1	....	....	22.00
Substance used for clarifying wine	1	....	....	33.20
Substance used for clarifying wine	1	....	....	59.30

[1] *Clayton (J. Soc. Chem. Ind., 1902, **21**, 670) considers this the best single chemical test.* In criticising Stelling's process, Kissling (*Chem. Ztg.*, 1898, **22**, 171) points out that pure gelatin is not completely insoluble in alcohol of 72%; while the non-gelatin consists of fat and other impurities beside the decomposition-products of gelatin. Nevertheless, Kissling considers that the method affords a partial indication of the value of a glue. He found the "non-gelatin" estimated by Stelling's method in 12 samples of commercial glue to range from 7.6 % in a skin glue to 23.2% in a very inferior specimen of bone glue.

mates these constituents by the following method A weight of 15 grm. of the sample is soaked overnight in 60 c.c. of water in a 250 c.c. flask. Next day the jelly is dissolved on the water-bath, and any loss by evaporation made up. The flask is then filled to the mark with 96% alcohol, and the liquid thoroughly shaken. After standing for 6 hours, 25 or 50 c.c. of the liquid is filtered off, evaporated to dryness, and the residue consisting of the non-gelatinous substances, dried at 100° and weighed. Stelling gives the figures, on p. 764 which though only of approximate accuracy, are comparable with each other.

Several other precipitation tests have been proposed. The one based on the precipitation by tannin is totally unreliable, because of the indefinite nature of the adsorption compound formed.[1] Trotman and Hackford (*J. Soc. Chem. Ind.*, 1904, **24**, 1072) proposed to separate the hydrolysed nitrogenous products from the true gelatin ("albumoses") by precipitation of the gelatin on saturation with zinc sulphate, and its subsequent estimation by the Kjeldahl method. The results of such a test, even if accurate, would be of little use in ascertaining the value of a glue.

As a rule, it is not advisable to waste time in making "chemical tests" on glues, for the physical tests recommended give more reliable and more useful information.[2]

Tensile or Binding Strength.—Among the numerous methods proposed to measure the "strength" of glue, only a few can be mentioned.

Weidenbusch's method consists in observing the breaking strain of a rod made of glue and plaster of Paris. J. Fels (*Chem. Ztg.*, 1897, pages 56, 70) describes this method as "uncertain."

Setterberg (*J. Soc. Chem. Ind.*, 1899, **18**, 55) dips strips of filter paper in definite solutions of glue, and determines their breaking strength after drying. This is also unreliable.

[1] Hans Trunkel (*Biochem. Z.*, **26**, 458; *Chem. Abs.*, 1910, **4**, 3091) states that 1 grm. of *freshly dissolved gelatin is completely precipitated by 0.7 grm. tannin. After the gelatin* has been in solution 24 hours, 0.4 grm. tannin will precipitate it, but the original condition returns upon warming. Any excess of tannin up to 3 parts of tannin to 1 part of gelatin is carried down. By prolonged washing of the tannin-gelatin complex with alcohol, 97 % but not all of the tannin is combination removed. He concludes that the gelatin-tannin *compound is an adsorption.*

[2] F. Gantter (*Z. anal. Chem.*, **32**, 413; abst. *J. Chem. Soc.*, 1895, **2**, 610) employs the following method for the valuation of hide-clippings for glue-making. A weight of 100 grm. of the clippings is boiled with 1 litre of water containing a few drops of sodium hydroxide until dissolved. Any sand or other mineral impurity is allowed to settle out, and fat removed in the usual way. The clear solution is then made up to 2 litres, 20 c.c. evaporated, and the residue dried at 105° weighed and ignited. This gives the total soluble organic matter. Another portion of 10 c.c. is diluted with 30 c.c. of water, neutralised with acetic acid, and treated with a solution of tannin till no further precipitation takes place. The liquid is then made up to 100 c.c., filtered, the excess of tannin removed by hide-powder, and the organic matter not precipitable by tannin determined in the filtrate by evaporating, weighing, and igniting as before. The difference between the total soluble organic matter previously determined and that not precipitable by tannio is regarded as gelatin.

Methods of testing glue have been based on the weight required to forcibly separate two surfaces of nickel-plated steel, wood, or stone which had been glued together by a solution of the samples. Rideal found wood and metal quite unreliable, and the results with stone-china far from constant. He considers that the rigidity of the stone blocks gives them an advantage over wood, the elasticity and compressibility of which often brings about the rupture prematurely.

As a rule, tests of this character are extremely variable, depending largely upon the personal equation, state of the weather, time and conditions of drying, etc. Specifications for glue for aeroplane propellers call for an average shearing strength of 2,400 lbs. per sq. in., with a minimum of 2,200. The glues used showed 2,500 to 3,000. Generally 130 test glue was used, though some plants did well with lower test glues. Much depends on workroom technique and temperature. (See "Modern Glues and Glue Handling" by Teesdale and Bezeau.) For details for certain "strength tests," see Army and Navy Specifications; also various Reports of the Forest Products Laboratories, Madison, Wisconsin. Further references are given in Alexander's and in Bogue's books, cited.

Interpretation of the Results of Tests.—Glue is used for a multitude of purposes, and each line of work has its special requirements. Experience has taught that glue of certain characteristics made from certain kinds of stock will, in certain cases, do the best work at the lowest cost. It is, therefore, best to test unknown glues against those which are actually doing the work, and give the ones which show up well, a trial under actual working conditions. If this is impossible, a laboratory test must be devised, in which the actual working conditions are exactly simulated; the more nearly this is done, the more valuable the test will be.

Regarding the selection of glue for particular purposes, a few special instances may be mentioned. In all cases, the working conditions must be considered, *e. g.* temperature of the work-room; time elapsing between the application of the hot fluid glue and the final pressing together of the surfaces to be joined, etc.

Wood Joints.—Most preferable are hide glues showing a jelly strength of 70 or over. While some bone glues, especially those made from acidified bone, answer admirably, in general they should be avoided. Lower viscosity of the working solution is preferable for

hard wood, on account of the better penetration; whereas with soft porous wood, higher viscosity is desirable.

Veneers.—Here viscosity is of great importance, and low-test hide or mixtures of bone and hide glue testing from 50 to 80, are most desirable. Higher test glues are apt to set too quickly unless the wood and room are warm. If worked on a veneering machine, freedom from foam is essential, for foam bubbles cause "blisters."

Sizing.—As most sizing is done with special machinery and mixtures, each case must be considered individually. Generally a free-flowing glue, free from foam, is required. If used to surface paper, the absence of grease, which makes spots or "eyes," is desirable. There should be no marked acidity or alkalinity, which might turn the shade of the colours with which the size is mixed.[1]

Gelatin.—*Technical gelatin* is used for such purposes as stiffening straw hats, finishing curtains, making hectograph composition, photographic emulsions, etc. As a rule, some kind of preservative is used in gelatin of this character, which may be properly described as a very strong, clear, light-coloured glue.

In judging of the suitability of gelatin for photographic purposes, Abney[2] considers: (1) The proportion of ash of minor importance, specimens of excellent quality sometimes containing 2.5%. (2) A good photographic gelatin will take up from 5 to 10 times its weight when soaked in cold water. (3) The solubility of commercial gelatin varies considerably. Thus Nelson's gelatin will dissolve in the ordinary "cold" water in warm weather, and scarcely sets at 24°, whereas Coignet's gold-label gelatin only melts at about 43.3°, and sets rapidly. For ordinary photographic emulsions, Abney recommends a mixture of hard and soft gelatins in proportions dependent on the weather, a good mixture containing 1 part of the former to 3 of the latter. (4) Fatty matters may be determined in the usual manner, or removed by skimming the solution of the sample or converting it into jelly and removing the top. (5) The colour of the solution, the tenacity of a jelly of known strength, and the presence of acid (which is sometimes present in sufficient proportion to be recognised by the taste) should also be noted.

[1] For further instances, see Alexander, *J. Soc. Chem. Ind.*, 1906, **25**, 158; also "Glue and Gelatin."

[2] Many manufacturers of photographic plates test out each boiling of gelatin by making small trial emulsions, and rejecting the lots of gelatin which do not give perfect results. In the absence of more definite knowledge on the subject, this appears to be the safest way; and it is interesting to note that boilings unsatisfactory to one manufacturer, may please another who uses a slightly different method of manipulation. Information regarding gelatin for photographic plates may also be found in *Eder's Handbuch der Photographie*, 5th Ed. **1**, 31, 36, 39, 1902. See also reference to work of Sheppard, *infra*.

Gelatin containing much chondrin is of inferior quality, and unsuitable for the preparation of photographic emulsions, the solution having less gelatinising power than that of pure gelatin. When present in notable proportion, chondrin may be detected by making a solution of the sample of gelatin in 10 parts of hot water. To this, a strong solution of chrome-alum is added, which in presence of much chondrin will cause the liquid to set to a jelly while still hot.[1]

Photographic Gelatin.—J. Alexander ("Glue and Gelatin," p. 208 *et seq.;* Chemical Catalog Co., 1923) gives particulars as to special tests made on gelatins to be used in photography; these are based on information kindly supplied by Dr. S. E. Sheppard, of the Eastman Kodak Co. of Rochester N. Y. There is always also made a practical working trial in an emulsion, which is naturally a crucial test. But the tests suggested show the probable behaviour of the gelatin during development and subsequent treatment.

Physical Tests

Jelly Strength.—High strength gelatin (130 test and upwards) is generally used. The jelly strength test is superior to the "melting point" test used to classify gelatins as "hard" or "soft."

Viscosity.—Using a 6 to 10% solution prepared under standardised conditions, the viscosity is determined (usually in centipoises) at 100° F. and 150° F.

Melting Point and Setting Point.—Various methods yield varying results. Using the apparatus described by Sheppard, Sweet and Scott (*Ind. Eng. Chem.*, 1920, **12**, 1007) 10% solutions of "soft" gelatins set at from 19° to 23° C; of "hard" gelatins, from 23° to 27° C. The corresponding melting points are 2° to 3° higher.

All these tests should be considered in the light of the ash analysis, and a determination of the hydrogen ion concentration (*p*H), which fixes the effective, as opposed to the total acidity. The usual *p*H of photographic gelatin is between 5 and 6, the limits allowable usually being 4 and 7. (The isoelectric point is about 4.7.) The physical properties of gelatin are greatly affected by the *p*H value, and also by the presence of aluminium salts.

[1] For the detection of traces of chlorides in gelatin ordinary methods fail. Luppo-Cramer (*Z. Chem. u. Ind. Kolloide*, 1909, **5**, 249–259) allows a small quantity of 10% gelatin solution to gel on a glass plate. On top is placed a drop of 10% $AgNO_3$, and in the course of a few hours an opalescent ring of gradually increasing density and about 1 or 2 cm. wide, forms about the drop. On exposure to light the whitish ring surrounding the drop becomes greyish-blue, whereas the colourless layer under the drop turns reddish-yellow.

The *clarity*, *colour*, *odour*, and *appearance* of the gelatin are always noted.

Chemical Tests

Moisture is usually limited to 8–15%. An over-dried gelatin generally gives trouble, whilst too moist a one is apt to spoil.

Ash.—The safe limit is 2%, though 3% is sometimes allowed. *Iron* and *copper* should not exceed 50 to 60 parts per million; *lead*, 50 parts per million. Al_2O_3 should be not over 0.2% on the dry weight of gelatin. *Sulphur dioxide*, determined by distillation into iodine, should not exceed 0.1%. Not more than a trace of *ammonia* should be present. The acidity or alkalinity is determined by titration of a 2% gelatin solution, using decinormal KOH or HCl, and phenolphthalein as indicator.

Reducing substances are usually detected in the emulsion test. Generally, photographic gelatin should not reduce cold ammoniacal silver nitrate solution in the dark, within 12 hours. *Grease* is found by the usual "brush out" test for "eyes" or "comets" (see p. 160) or the finely powdered gelatin may be extracted with benzene. Only a negligible amount should be present.

Mucin, Etc.—A 2 to 5% solution should give no precipitate when acidified with acetic acid (to about 3%), or when 1 to 2% of alum or of chrome alum is added.

Recent investigations have shown that the photographic properties of gelatins largely depend on the presence of minute traces of sulphur-containing organic substances, which appear to form silver sulphide by reaction with the silver halides. The chief substances which occur naturally are mustard oils and the thioureas derived from them, and cystine or substances yielding cystine. The former are more active, and are probably derived from plants eaten by the animals from which the gelatin was made. (See S. E. Sheppard, "Photographic Sensitivity; A Colloid Chemical Problem," *Third Colloid Symposium Monograph*, 1925; also S. E. Sheppard, "Photographic Gelatin," *Photographic Journal*, 1925, **65**, 380.)

Gelatin photographic emulsions are of very variable composition, and occasionally it is desirable to examine them quantitatively.

For the estimation of the silver, the sample should be dissolved in or diluted with water, and digested with excess of dilute nitric acid at

100° for some hours. The precipitate will probably consist simply of silver bromide, but may also contain chloride and iodide.[1]

Addition of silver nitrate to the filtrate causes a precipitation of silver bromide, etc., corresponding to any potassium bromide present in the original emulsion. For the estimation of the water, the emulsion is dried at 100°. Air-dried gelatin emulsions generally lose from 8 to 15% of water at 100°. The proportion of gelatin in the sample is ascertained by subtracting from the dry residue the amounts of silver and potassium salts previously found.

Haloid salts of potassium and silver may, with gelatin, be regarded as the normal constituents of gelatin emulsions. Some samples contain foreign ingredients, for the detection of which a large quantity of the emulsion should be pressed between canvas, and 50 grm. macerated in cold water for 12 hours. The soluble salts diffuse into the water, and may be detected therein by ordinary methods. For the detection of alcohol, the original sample should be distilled, while phenol and thymol may be detected by the odour developed on warming with sulphuric acid. Salicylic acid may be detected by precipitating the aqueous solution with three volumes of alcohol, evaporating the filtrate to a small bulk, agitating with ether, treating the ethereal layer with dilute sodium hydroxide, faintly acidifying with hydrochloric acid, and adding ferric chloride solution, which will give a violet coloration if salicylic acid is present. Excess of silver nitrate may be detected by neutral potassium chromate, and may be determined by titration with standard sodium chloride, employing the chromate as an indicator.

For the analysis of collodio-gelatin emulsions, the sample should be treated with a large quantity of water, which precipitates the silver bromide and pyroxylin. After drying at 100°, the precipitate is weighed, moistened with nitric acid, and ignited, the loss of weight being the pyroxylin. The aqueous filtrate contains the gelatin. The acetic acid is determined by titrating a portion of the liquid, and the alcohol by distillation of the neutralised portion. Ether does not occur in this last form of emulsion.

Edible Gelatin.—Only clean gelatin, free from harmful substances, and properly made from carefully selected raw material, under sanitary conditions, should be sold for food purposes. Food gelatin is used in the manufacture of confectionery, charlotte-russe, ice-

[1] Where great exactness is not required the silver chloride may be dissolved out by ammonium carbonate, and the bromide separated from iodide by strong ammonia.

cream, "jellies," and for numerous other culinary purposes, besides serving to coat pills and make gelatin capsules and globules used in pharmacy.[1] In some case, *e. g.* making "marshmallow biscuits," it is desirable to have a good *quantity* of gelatin in the final product, and low-test gelatins are used.

The function of gelatin in ice cream, besides adding "body," is to inhibit the formation of sharp ice crystals, thus giving a smooth velvety *feel* to the tongue. Gelatin also acts as a protective colloid, inhibiting the coagulation of the casein, and preventing the formation of large greasy curds, which are not easily digestible. Dahlberg, Carpenter and Henning (*Ind. Eng. Chem.*, 1928, **20**, 397 and 516) have made special tests on gelatin in ice cream. M. Briefer and J. H. Cohen (*Ind. Eng. Chem.*, 1928, **20**, 408) have studied the relation between the physical properties of pure food gelatins, and their practical uses. There are notable differences in behaviour between gelatins made from limed and from acid-plumped stock, especially in the effects of variations in *p*H.

J. C. Kernot and J. Knaggs (*Rept. of Food Investigation Board* for 1927, *Dept. of Ind. and Sci. Research*, pp. 68–82) also found that the method of preparing the stock exerts an important influence on the gelatin; that made by alkaline treatment of the stock gives better emulsification and also shows a higher percentage of free amino groups than gelatin made from acid-treated stock. It is also interesting that they observed the relative emulsion-forming capacity of oils to be fish oils > vegetable oils > mineral oils, which is also the order of relative unsaturation of the valencies, mineral oil, naturally, being practically saturated.

Working Directions for Viscosity and Jelly Strength Determinations of Edible Gelatin

The following detailed instructions were worked out by W. D. Richardson, G. R. Underwood, L. M. Tolman and J. H. Cohen, a Committee of the National Association of Gelatin Manufacturers (U. S. A.):

The equipment described in the accompanying Official Standard Methods for Determining Viscosity and Jelly Strength of Edible Gelatin is to be used in all cases.

The following is the procedure for a given sample. As a matter of convenience, it is suggested that routine samples are to be scheduled

[1] See U. S. Pharmacopœia and British Pharmacopœia.

about 4 minutes apart, with particular references to the soaking period and viscosity pipette, and that arrangements are made so that each individual sample is handled according to the following method:

1. Grind flake or wheel gelatin to four mesh or finer. If the gelatin is in the form of thin sheets, either grind or cut these to convenient size for weighing.

2. Weigh out 7.50 ± 0.025 gms. of the sample prepared as above and transfer to the standard bottle. Add 105 ± 0.25 gm. of distilled water at 14–16° C., while stirring with a pointed metal rod. Close bottle with the standard perforated rubber stopper, made by puncturing with heated wire.

3. Place in water bath at 10.0° C., and soak one to three hours, according to the fineness of the sample. Place and remove samples with the least possible disturbance of the temperature of the bath.

4. At the end of the soaking period, place the sample in a water bath at 45.0° C. for four minutes. Then place in water bath held at 65° ± 1.0° C. The sample is to be brought to 61.0° C., in a period not to exceed fifteen minutes. Near the end of this period the perforated stopper is replaced by another perforated one carrying a thermometer. The bottle is then swirled carefully to make the temperature uniformly 61.0° C. Any motion that will produce violent agitation of the solution is to be avoided.

5. The sample is then poured into the viscosity pipette as quickly as possible without entrapping air. Enough of the solution is poured into the viscosity pipette to bring its level about 1 cm. above the upper mark of the pipette, while the finger of the free hand covers the capillary end. The bottle containing the rest of the solution is placed under the exit tube of the pipette. The special centigrade thermometer furnished with the apparatus (which may be the standard F.A.C. 65.0° titer thermometer made by the Taylor Instrument Company) is lowered and raised until it registers and maintains a temperature of 60.0° C., when its bulb is about midway between the top and bottom of the bulb of the pipette. The thermometer is then removed, and the level of the solution adjusted to the upper mark, reading the bottom of the meniscus. When not in actual use, the thermometer is placed to nearly its full length in water of a temperature slightly above 60.0° C. It is wiped dry before use. The time required for the 100 c.c. of solution to pass through the capillary tube of the viscosity pipette is obtained by

simultaneously starting the stop watch and withdrawing the finger from the capillary end of the pipette and then stopping the watch when the level of the solution has reached the lower mark on the pipette. The container is then closed with the original perforated stopper and the sample used for the jelly strength determination. The pipette, after each determination and also after the apparatus has been idle for any length of time, is washed out with about 25 c.c. of water heated to 62.0° C., and allowed to drain thoroughly. Occasionally, when necessary, the pipette should be thoroughly cleaned with bichromate and sulphuric acid. When not in use the funnel end of the viscosity pipette should be covered to prevent the entrance of foreign matter. The viscosity is to be expressed in millipoises, calculated according to the formula appearing on the certificate of calibration accompanying each pipette and as described in the circular, using sp. g. 1.002 in place of 1.020.

6. The sample from the viscosity determination is immediately closed with a perforated stopper and placed in a water bath at 45° ± 1.0° C. It should remain in this bath until it has cooled to 45° ± 1.0° as registered by a thermometer at the centre of one of the bottles. This should take not over 20 minutes. The finger is then placed over the hole in the stopper and the bottle inverted several times to mix in thoroughly any water of condensation, but without violent agitation of the solution. The bottle is then placed in the 10.0° C. water bath.

7. When the standard chill bath of the Association is used, samples are to be placed and removed with the least possible disturbance of the temperature of the bath. The bath is maintained at 10.0° ± 0.05° C., and the table must be perfectly level. The schedule should be planned on a basis of seventeen hours in the 10.0° C. bath, and no individual sample should stay in less than sixteen hours, nor more than eighteen hours.

8. At the end of the chilling period the sample is to be tested by the Bloom Gelometer. The Gelometer should be adjusted to give exactly a 4 mm. depression which should be checked daily by means of the gauge furnished with the instrument. It should deliver the shot at the rate of 200 ± 5 grm. in 5 sec. when the arm is on the second dog. The hopper should contain 800 grm. of polished No. 12 chilled lead shot. The rate of delivery and weight in the hopper should be checked at least weekly. The directions for operation

furnished with the Gelometer must be carefully followed in every detail. Note especially—

(a) Cool the plunger tip by immersing in 10.0° C. water and drying before the first sample is tested or after any interruption in the testing, unless tests are conducted in a chill room at 10.0° C.

(b) The contact surface of the plunger must be parallel to the top of the elevating platform and touch the jelly at the center of the bottle. If all adjustments are properly made, the plunger will not swing when the bottle is lowered after testing.

(c) It is very important that all foam be removed from the centre of the surface before setting the bottle in the 10.0° C. bath.

9. Report jelly strength in grm. (Grades 1–14) by the Bloom Gelometer.

In food gelatins the purity, colour, clearness and jelly strength are of prime importance, and since they are usually of very high test, the "finger test" of the jellies is made on solutions of from 3 to 10 grm. of gelatin to 100 c.c. of water.

Food gelatin may be tested[1] for the usual metallic impurities, preservatives, etc. W. B. Hart (7th Int. Cong. of Appl. Chem.) reports finding as much as 104 mg. of copper per kilo. in gelatin that was used to garnish pressed beef. R. Krzizan (*Z. offentl. Chem.*, 1909, **15**, 31; *Chem. Abs.*, **3**, 1430) also reports finding 0.014–0.026% in two samples of gelatin. Owing to the careful chemical control exercised by manufacturers to-day, which is, furthermore, checked by official tests, gelatin is now one of the purest of manufactured products.[2]

The estimation of sulphurous acid and sulphites in food gelatin is of particular interest, because these are the antiseptic substances more frequently sought for and found.

For a qualitative test William Lange (*Arb. kais. Gesundheitsamt*, **23**, 144; *Chem. Abs.*, 1909, **3**, 2989) treats 5–10 grm. of gelatin in a wide-mouthed 150 c.c. flask with 30–40 c.c. of water, warms slightly, and adds 5–10 grm. H_3PO_4 (sp. gr. 1.15). The flask is loosely stoppered with a cork from which is appended a strip of potassium iodide paper. On warming, sulphur dioxide produces a blue colour on the paper.

[1] Many tests are seriously affected by the presence of gelatin or similar protective colloid, in the presence of which most precipitates are colloidal or fail to form. The gelatin should therefore be burned to an ash, or the impurities sought dialysed out. See J. Alexander, *J. Soc. Chem.*, 1909, **28**, 285.

[2] Modern medical research indicates that traces of many things harmful in quantity are essential to health. Copper apparently serves in the liver. "Purity" pushed to an extreme may deprive us of essentials; *e. g.* fluorine is needed in the teeth.

The gravimetric[1] method is generally used in the estimation of sulphur dioxide in gelatin. 200 c.c. of 20% phosphoric acid are added to a solution of 10 grm. of gelatin in 10 c.c. of water, and 50 c.c. are distilled over, in a current of pure carbon dioxide into a solution of iodine or bromine. The sulphur dioxide that distils over is oxidised to sulphuric acid; which is precipitated and weighed as barium sulphate.

The accuracy of this method, especially for small percentages of sulphur dioxide, has been frequently attacked. Alexander (*J. Amer. Chem. Soc.* 1907, **29**, 783) found, that when tested by the gravimetric method, skin, bone and horn pith, obtained from animals freshly slaughtered under government supervision, showed apparent sulphur dioxide, evidently derived from the oxidation of volatile sulphur compounds which distil over. His results were confirmed by William Lange (*loc. cit.*), who states that the error due to this source is from 0.0007 to 0.0012%. A Mueller (*ibid.*, **34**, 164; *Chem. Abs.*, 1910, **4**, 3083) found apparent sulphur dioxide in all the gelatin he examined, but observed that the 0.014–0.082% found in the finest white gelatin did not interfere with the growth of bacteria, which would indicate that it is adsorbed or combined in some manner. The Official Association of Agricultural Chemists recommend (*U. S. Dept. Agri. Bur. Chem. Bull.* 107, rev. page 113, 1910) that in the case of meat food products, "mere traces" of SO_2 be ignored.

E. Gudeman, (*J. Ind.* and *Eng. Chem.*, 1908, **1**, 81) also criticises the gravimetric method, and describes a modified form, using steam distillation, by which he claims to obtain very accurate results. L. Padé (*Ann. Chem. anal.*, 1908, **13**, 299; *J. Soc. Chem. Ind.*, 1908, **27**, 914) gives details of a modification of the gravimetric method.

The Preservatives Determination Committee of the Chemists of the Manufacturing Confectioners' Alliance and of the Food Manufacturers' Federation have considered the determination of sulphur dioxide in food products, including gelatin (see *Analyst*, 1928, **53**, 125). They describe apparatus and procedure for both volumetric and gravimetric estimation, both using iodine to oxidise the SO_2 into SO_3. Steam distillation is recommended for gelatin, and hydrochloric acid instead of phosphoric acid (25 c.c. concentrated HCl and 250 c.c. of air-free water to 25 grm. of gelatin). The same samples

[1] The volumetric estimation of SO_2 by receiving the distillate into standardised iodine, and titrating with sodium thiosulphate to determine the amount of iodine consumed, is quite inaccurate, lowing to the presence in the distillate of reducing substances other than SO_2. Free SO_2 is estimated by distilling without the addition of phosphoric acid.

tested in several different laboratories both by these methods and the Monier-Williams method (*Analyst*, 1927, **52**, 343; 415; also Ministry of Health Report on Public Health & Medical Subjects, No. 43) gave results which varied materially from each other (approximately 4 or 5%).

Isinglass[1] (Ger. *Hausenblase*, literally sturgeon's bladder) is a variety of collagen prepared from the "sound" or swimming bladder of the sturgeon and other fishes. It should be white, with a yellowish tinge, opaline or semi-transparent, fibrous, and tenacious. When pure, isinglass has no odour, and but a faint taste. It contains from 15 to 20% of moisture.

According to Mülder, isinglass contains: Carbon, 50.76; hydrogen, 6.64; nitrogen, 18.32; and oxygen and sulphur, 24.69%.

Isinglass consists of nearly pure collagen. It is not gelatin, but is converted into that substance by heating with water. It consists of fibres or threads which, when immersed in cold water, swell up but retain their organised structure, and it is to this property that the use of isinglass for clarifying wine, beer, and other liquids is partly due. The best qualities of isinglass dissolve almost entirely in boiling water. The solution gives a remarkably strong jelly on cooling.

Russian isinglass is the kind most valued, its solution having a higher viscosity than any other variety. It is obtained from various varieties of sturgeon, *Acipenser stellatus* (the seuruga), *A. ruthenus* (the sterlet), and *A. güldenstadtii* (the ossetr), which inhabit the Volga, Caspian and Black Seas, and the Arctic Ocean. The finest are Astrakhan and Taganrog (Samovey) isinglass, which is prepared by steeping the sounds in hot water to remove mucus, cutting them open and drying the inner membrane, and then removing the outer membrane by rubbing or beating.

The unopened sound is called "pipe," "purse," or "lump isinglass"; when opened or folded, "leaf or honey-comb isinglass"; when folded and dried, "book isinglass"; and when rolled, "ribbon isinglass."

Penang isinglass is made from various unknown fish. Brazilian isinglass, sometimes called "Cayenne isinglass," is obtained from *Silurus Parkerii*, and occurs in leaves an inch or more in thickness. Rat's tail isinglass is made from the swimming bladder of the cod (*Morrhua vulgaris*), the hake (*Phycis americanus*) and other fishes; it is opaque, and incompletely soluble in water.[2]

[1] It is a common popular error to call the mineral mica, "isinglass," probably because its sheets are transparent and flexible.

[2] Interesting information on the origin, preparation, and applications of isinglass will be found in the *J. Soc. Chem. Ind.*, 1887, **6**, 764.

Isinglass which has been bleached by sulphurous acid generally retains traces of sulphates, and hence the solution gives a precipitate with barium chloride.

Isinglass is not infrequently adulterated with bone-gelatin, gut, and inferior kinds of fish-gelatin.

On treating Russian isinglass with hot water the substance swells uniformly, producing a whitish opaline jelly, which gradually dissolves entirely. Gelatin, on the contrary, swells irregularly, and gives a nearly transparent solution.

On ignition, Russian isinglass usually leaves from 0.4 to 0.9% of reddish ash, containing a little calcium carbonate. Gelatin often yields some white ash, consisting of calcium phosphate or carbonate, with traces of chlorides and sulphates. If the gelatin is not specially purified, the ash may be over 1–1.5%.

On treating isinglass with hot water, any admixture of ordinary intestinal membrane will be left insoluble, or, at any rate, not more than 30% of the adulterant will pass into solution. Inferior fish-gelatins leave from 20 to 30% of insoluble residue, and give solutions of a very strong and disagreeable odour.

F. Prollius (abst. *J. Chem. Soc.*, 1884, **45**, 647) has examined a number of samples of isinglass and fish-gelatin from different sources. The insoluble matter was determined by weighing the residue obtained on treating the sample with hot water. The gelatinising power was ascertained by dissolving 1 part of the sample in 90 parts of water, filtering, and determining the viscosity of the resulting solution. The table on p. 778 shows the results obtained.

Prollius recommends the microscopic examination of isinglass as an indication of its purity. Isinglass exhibits a very pronounced fibrous structure, which is never observed in the case of ordinary gelatin.

The commoner kinds of isinglass, especially coarse Brazilian, are employed for clarifying wine, etc. *Beer-finings* are usually prepared by treating isinglass or fish-gelatin with sour beer, or with acetic or sulphurous acid. The last reagent is preferred by brewers, since the antiseptic properties of the sulphurous acid are of value. One pint of finings containing ⅛ oz. of isinglass, "cut" by sulphurous acid, should fine a barrel (36 gallons) of beer. The finings are not actually dissolved but simply softened or "cut" by the acid. The clarifying action appears to be purely mechanical, the particles causing turbidity becoming entangled in the gradually sinking network of gelat-

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Source of isinglass	Ash, %	Water, %	Residue insoluble in hot water, %	Viscosity, seconds
Astrakhan, from Schmidt and Dihlmann, Stüttgart	0.20	16.0	2.8	507
Astrakhan, from a collection	0.37	18.0	0.7	485
Astrakhan, fine iridescent Russian quality, Tübingen collection	1.20	17.0	1.0	500
Astrakhan, Russian, from Gehe, of Dresden	0.80	19.0	3.0	491
Astrakhan, in laminæ, from Gehe	0.50	19.0	0.4	480
Astrakhan, in threads (Hamburg threads)	0.40	17.0	1.3	477
Hamburg isinglass	1.30	19.0	2.3	470
Hamburg isinglass, another quality	0.13	19.0	5.2	....
Rolled northern fish-bladder	3.20	......	10.8	467
Icelandic bladder	0.60	17.0	21.6	463
Indian isinglass	0.78	18.0	8.6	437
Yellow, unknown quality	2.30	17.0	15.6	360

inous matter. Hence an actual solution of any kind of gelatin would be useless for this purpose.[1]

Isinglass is employed by cooks for thickening soups, jellies, etc., but in this case its clarifying properties are not called into play, and hence gelatin is generally used under the name of "patent isinglass."

Isinglass, though free from chondrin, is not suitable for the production of photographic films, on account of its ready solubility and inferior tenacity.

Liquid Fish Glue

A detailed description of the manufacture and testing of liquid fish glues (and Isinglass) is given by Donald K. Tressler in "Marine Products of Commerce," Vol. 25 (Chemical Catalog Co., 1923). The three classes of stock used for making liquid fish glues are skins, heads, and mixed waste (includes bones and trimmings). The washed stock is usually heated in the presence of some acetic or other moderately strong acid which facilitates hydrolysis. Evaporation is usually effected by a steam coil, open to the atmosphere, revolving in the dilute glue liquor. For photo-engravers' use, first-run skin glue is used, which is kept free from the large amounts of preservatives (phenol, cresol, boric acid, etc.) and essential oils

[1] High-test gelatin, broken in small pieces, is also used for fining, and is to be recommended from the standpoint of cleanliness and purity.

(cassia, wintergreen, sassafras, peppermint), which help to cloud the other grades in which they are used. The usual commercial grades of fish glue contain from 50 to 55% of dry glue, and weigh about 9.5 to 10 pounds per gallon (American). They contain about 1% of preservatives, 0.2 to 0.4% of essential oils, and sometimes some white pigment (ZnO, lithopone, TiO_2) or some methyl or ethyl alcohol.

A sample of skin glue showed the following composition (*Chem. Age.* 1921, **29**, 173–5):

Ash, in dry matter	0.96%
SiO_2	12.7
CaO	10.5
MgO	trace
K_2O and Na_2O	13.9
SO_3	34.0
P_2O_5	24.9
Cl	3.2
Fe_2O_3	trace

R. H. Bogue (*Met. Chem. Eng.*, 1920, **23**, 105–9, 154–8) gives the following figures on a sample of fish glue (kind not stated);

Protein N	33.4%
Proteose N	42.3
Peptone N	21.9
Amino acid N	2.4

The purified protein, obtained by precipitating with 95% alcohol, redissolving and reprecipitating four times, showed the following analysis:

Ammonia N	5.15%
Melanin N	1.12
Cystine N	trace
Arginine N	13.80
Histidine N	2.04
Lysine N	8.58
Amino N in filtrate	60.20
Non-amino N in filtrate	9.66

The large amount of cold-water-soluble proteoses and peptones, and the relatively small amount of protein, explain why fish glue is liquid at ordinary temperatures. It resembles in composition low grade bone glue. (See pp. 156, 168.) Many liquid glues are not fish glues; some are made by fluidifying animal glues.

Tressler recommends the following tests for liquid glues:

Gel Point.—Chill in a test tube, noting when flow ceases. Then warm slowly, noting melting point. The average of the two gives a close approximation to the true gel point.

Viscosity.—Tressler recommends using the Stormer viscometer, substituting a 500 gram weight for the regular 100 gram capsule; but any viscometer can be used, which will operate with so viscous a solution. The viscosity should be taken over a range of temperatures, and plotted against the temperature; extrapolating to infinite viscosity, shows where the gel point is.

Moisture is determined by heating for about 5 hours at 100°.

Speed of set is determined by comparing the speed of skin formation, using known liquid glues for comparison.

Ash is best determined by using the sample from the moisture determination moistening with standard calcium acetate solution, and igniting as per the method adopted by the Association of Official Agricultural Chemists (U. S. Dept. Agr., Bureau of Chem., *Bull.* 107, p. 21, 1907). Chlorides usually indicate high hygroscopicity; the best glues should not have over 0.02% Cl as chlorides.

Drying properties are tested comparatively, against some known glue, by observing the time needed to dry a 0.2 cm. thick layer on a glass plate, at e. g. 25 degrees and 70% humidity. Best grades give strong, brittle films. If the film powders easily, the glue is not useful in many kinds of work.

Hygroscopicity is determined by noting the gain in weight of the dried film under fixed conditions. Thus, the film is placed over 80% sulfuric acid at 25 degrees. Slightly hygroscopic glue becomes flexible; but a glue which becomes sticky and molasses-like, is of little use for joints.

Joint strength may be determined by gluing tests, as with animal glues (see p. 170).

Colour and *permanence* of properties must also be considered.

Size is a glue or gelatin solution employed in paper-making, distemper-painting, and for weighting and imparting stiffness and gloss to cotton goods, etc. It is generally made by boiling hide pieces, rabbit skins, etc., adding a preservative (zinc sulphate or cresol), and allowing the liquor to gel. The expense of drying and re-solution are avoided, but, on the other hand, much water has to

be packed and freighted, so that size is usually used at or near the point of its production.

Bone-size is pale, clear, and forms solid, semi-transparent cakes or masses. Its adhesive power is inferior to glue-size.

Glue-size is a dark brown, semi-fluid, very adhesive mass. It frequently contains hairs and animal refuse, and it often gives strongly the reactions for chondrin (pp. 190, 192), a substance which tends to produce foaming.

When exposed to warmth and moisture, size is liable to mildew, with disastrous results to the goods on which it has been applied. Salts of magnesium and zinc and other substances of antiseptic properties are largely employed to prevent this change. Common salt is sometimes present in large proportion.

The feel and apparent tenacity are not reliable guides in judging of the quality of size, and the sp. gr. is equally misleading.

In assaying size, a portion should be dissolved in water, and a quantity of the solution, representing a known weight of the sample, should be evaporated to dryness in a shallow dish to ascertain the proportion of solid matter. Another portion of the solution should be treated with sulphuric acid, evaporated to dryness, and the residue ignited to obtain the sulphated ash, which will represent the mineral additions to the size.

Casein-glue is prepared by coagulating separated milk, as free as possible from fat, with dilute hydrochloric acid. Or the milk may be allowed to sour, in which case the lactic acid which forms causes curding. Argentine and French caseins are self-soured, and have characteristic properties. The curd is well washed, pressed, and dissolved in a strong solution of borax or sodium hydroxide; or commercial casein is used. The resulting viscous fluid has strongly adhesive properties, and is well-suited for use by joiners, bookbinders, etc.

Gluten- or albumin-glue is prepared from partially decayed gluten, obtained in the manufacture of starch from wheaten flour.

Blood albumin forms the basis of some water-proof glues.

Liquid glue is prepared by dissolving 5 parts of glue in its own weight of water, and adding 1 part of nitric acid of 1.31 sp. gr. When the evolution of nitrous fumes is at an end, the liquid is cooled. By the above treatment, the glue loses its character of gelatinising without its adhesive properties being impaired. A preferable preparation is obtained by dissolving gelatin or fine glue in mod-

erately strong acetic acid, with the aid of heat, and then adding to the solution some powdered alum and one-fourth of its volume of alcohol.[1]

Coagulin is a strong solution of gelatin in concentrated acetic acid. It becomes fluid on warming, but gelatinises partly on cooling. Many adhesives made of casein, albumins (plant or animal), starches, dextrins, etc. are, by similitude, called *glues*. References to some of these are to be found in publications of the Forest Products Laboratory, in books on glues and adhesives (*e. g.* Bogue, Alexander), and in the literature and patents.

Gelatin Substitutes

Various lichens and sea-weeds yield a highly gelatinous product on boiling with water, and some of them are employed as articles of food and in medicine.

The following table (from Thorpe's *Dictionary of Applied Chemistry*) shows the composition of various *British algæ:*

Alga	Water, %	Nitrogen in dry substance, %
Chondrus crispus, bleached	17.92	1.534
Chondrus crispus, bleached	19.79	1.485
Chondrus crispus, unbleached	21.47	2.142
Chondrus crispus, unbleached	19.96	2.510
Gigantina mamillosa	21.55	2.198
Laminaria digitata (dulse tangle)	21.38	1.588
Rhodymania palmata	16.56	3.465
Porphyra laciniata	17.41	4.650
Sarcophyllis edulis	19.61	3.088
Alaria esculenta	17.91	2.424

From the above results it is evident that the jellies from these sources, as a rule, contain but little nitrogen, and resemble gelatin merely in their physical characters.[2]

[1] Knaffl prepares a superior liquid glue by treating 3 parts of glue with 8 parts of water, 0.5 of hydrochloric acid, and 0.75 of zinc sulphate. The whole is heated for 12 hours to a temperature not exceeding 85°. The product keeps for a long time and is largely employed for joining horn, wood, and mother-of-pearl.

[2] **Cetrarin or Lichenin** is the gelatinous substance of *Cetraria Islandica*, a native of the north of Europe, commonly known as Iceland moss or Iceland lichen. The lichen was official in the British Pharmacopœia of 1885, which described it as almost odourless when dry, but having a feeble sea-weed-like odour when moistened with water. The taste is *mucilaginous* and bitter. *A strong decoction gelatinises on cooling. By prolonged* boiling with dilute sulphuric acid, the jelly from Iceland moss yields a crystallisable sugar, having an optical activity of $[\alpha]_D = +46.85°$. *Lichenstearic acid*, $C_{43}H_{78}O_{13}$, and *cetraric acid*, $C_{30}H_{30}O_{12}$, have been obtained from Iceland moss (abst. *J. Chem. Soc.*, 1890, page 600). Cetraria is omitted from the British Pharmacopœia, but is official in most foreign Pharmacopœias. It is described as demulcent, nutritious, and slightly tonic. In Iceland, the bitter principle is removed by mashing in cold water and sodium carbonate, and the lichen then made into jelly, or dried, ground, and made into bread.

Gelose is a gelatinous substance contained in *Gelideum corneum*, an alga known as Chinese moss or Japanese isinglass, Japanese gelatin, or vegetable isinglass. The sea-weed contains only traces of soluble matters, but swells up in cold water and dissolves wholly in boiling water, with the exception of 2 to 3% of nitrogenised corpuscles. The solution coagulates, on cooling, to a colourless translucent jelly. Payen prepared gelose by treating *Gelideum corneum* (or certain other algæ) with cold dilute acetic acid, water, and dilute ammonia; washing thoroughly, dissolving the substance thus purified in boiling water, and drying the jelly which formed on cooling.

Gelose occurs in commerce in bundles of long, very slender threads, resembling isinglass. Gelose has 10 times the gelatinising power of isinglass, and will set to a jelly with 500 times its weight of water. It is not, however, a suitable substitute for isinglass, since the m. p. of the jelly is above the temperature of the mouth. The aqueous solution of gelose is precipitated by alcohol. After drying, gelose is insoluble in cold water, dilute acids or alkalies, and Schweitzer's ammonio-cupric solution. Gelose loses its property of gelatinising when heated with water under a pressure of six atmospheres, or when boiled with dilute acids (including acetic acid), and the product reduces Fehling's solution on boiling. When heated with nitric acid, gelose yields mucic and oxalic acids. A 10% aqueous solution of gelose exhibits a specific rotatory power of $-4.25°$; but by boiling the acidified solution, the liquid gradually acquires a nearly equal dextro-rotation, and is no longer precipitated by alcohol. On the other hand, by treating gelose with water at 100°, Porumbaru (*Compt. rend.* **90**, 1081) obtained a *lævo*-rotatory sugar containing $C_6H_{12}O_6,H_2O$.

The gelatinous principle of *agar-agar*, an edible sea-weed found in Japan, Malacca, Borneo, Ceylon, etc., is probably identical with gelose. It is known commercially as Japanese gelatin.

Alginic Acid or **Algin** is a gelatinous substance present in certain sea-weeds, especially *Laminaria*. It was first isolated by E. C. C.

Carrageenin is the gelatinous substance contained in *Chondrus crispus* or Irish moss, a sea-weed which is only exposed at low spring tides. It contains about 80% of dry matter, containing 1½ to 2% of nitrogen. The greater part of the moss consists of carrageenin, a gelatinous substance apparently allied to pectin. The decoction or jelly is employed as a demulcent and emollient in pulmonary affections, etc., and is also used as a substitute for isinglass. It also finds employment as a size, and, to a limited extent, for thickening colours in calico-printing and for stiffening silk. A thick mucilage of Irish moss, suitably scented, is employed as "bandoline" or "fixature." On the west coast of Ireland, where it abounds, the moss is used as an article of food. Carrageen moss is official in many foreign Pharmacopœias.

Stanford (*J. Soc. Chem. Ind.*, **3**, 297; **4**, 518, 595; *J. Soc. Arts*, 1884), who has observed that the whole of the alkaline salts, together with a considerable quantity of extractive matter containing dextrin and mannitol, can be extracted from the Laminaria or "dulse tangles" by simple maceration in cold water. This treatment removes about 33% of the air-dry weed, of which from 20 to 22% consists of salts of potassium, sodium, and magnesium, including the whole of the iodine. The residue insoluble in cold water consists substantially of the above-mentioned nitrogenised substance, alginic acid, together with the algic cellulose or algulose which represents the cellular fabric of the plant. The alginic acid or algin is removed by digestion in a hot dilute solution of sodium carbonate, which dissolves it as sodium alginate, leaving the algulose in an extremely fine state of division, which renders it very difficult to remove by filtration. On treating the filtrate with hydrochloric acid, the alginic acid is precipitated as an amorphous substance of light amber colour, which is washed and bleached.[1]

Several patents have been obtained for obtaining "*tang-acid*" and other organic products from *sea-weed* (see *English Patents*, 1896, No. 11538; 1898, Nos. 12275 and 12277).

Alginic acid is stated to have the formula $C_{76}H_{80}O_{22}N_2$. When precipitated by adding a mineral acid to the solution of its salts, alginic acid forms a very gelatinous precipitate, which when dry resembles albumin or horn. It has a sp. gr. of 1.5. Alginic acid is insoluble in either hot or cold water.

Alginic acid acts on sodium carbonate, with evolution of carbon dioxide and formation of *sodium alginate* or soluble algin.

Soluble algin dissolves in water to form a very viscous solution. Stanford states that the viscosity of algin is 14 times that of starch, and 37 times that of gum-arabic. Solutions of sodium alginate are precipitated or coagulated by alcohol, acetone, and collodion, but not by amylic alcohol, ether, glycerin, or sugar. Alginic acid is precipitated from the solution of its sodium salt by most mineral acids, and by picric, oxalic, tartaric, and citric acids. A 2% aqueous solution is rendered semi-solid by acidifying it with hydrochloric acid. On the other hand, no precipitation occurs on treating a

[1] Stanford gives the following analyses of *Laminaria*. In addition to the three main constituents, the aqueous solution contains salts, mucilage, and mannitol, while in the sodium carbonate solution a modified dextrin is present.

	Water	Alginic acid	Cellulose	Salts and undetermined matters
Laminaria digitata, stem	37.04	21.00	28.20	13.76
Laminaria digitata, frond	44.00	17.85	11.00	27.15
Laminaria stenophylla, stem	34.50	25.70	11.27	28.53
Laminaria stenophylla, frond	40.02	24.00	15.06	20.86

solution of sodium alginate with acetic, formic, benzoic, succinic, carbolic, tannic, arsenious, or boric acid. Soluble algin yields no precipitate with silicates of alkali metals, chromates, permanganates, tungstates, or molybdates. It is not precipitated by hydrogen peroxide, chlorine, bromine, or iodine.

Sodium alginate gives no precipitate with the alkali hydroxides or with ammonia, but insoluble alginates are thrown down by lime water and baryta water. Precipitates are also produced by most metallic salts, some of the alginates formed being of curiously complex composition. No precipitates are produced by salts of magnesium, the alginate of this metal being soluble. By bringing magnesia or magnesium carbonate in contact with alginic acid and water, the two insoluble bodies react to form soluble *magnesium alginate*.

Solutions of sodium alginate are distinguished from those of albumin by not coagulating on heating and by yielding no precipitate with mercuric chloride, potassium ferrocyanide, or tannic acid; from mucin, by not being precipitated by acetic acid; from gelatin, by giving no precipitate with tannin; from starch, by yielding no blue colour with iodine; from gelose, by containing nitrogen and not gelatinising on cooling. Sodium alginate differs from dextrin, gum-arabic, gum-tragacanth, and pectin, by its insolubility in dilute alcohol and dilute mineral acids.

A number of ingenious applications of alginic acid and its salts have been proposed by Stanford (*J. Soc. Chem. Ind.*, 1885, page 518). Some of these salts are likely to prove valuable as envelopes for certain medicines—such as mercury, bismuth, etc.—since they pass through the stomach unaltered, and hence delay the absorption of the medicine till it has reached the duodenum.

Funori is a sizing material made in Japan mainly from a seaweed *Gloipeltis coliformis* and *G. intricata*. The algæ are washed, and then bleached and dried in the sun. Funori dissolves in boiling water. (See D. K. Tressler, "Marine Products of Commerce," p. 142; Chemical Catalog Co., 1923.)

Chondrigen and Chondrin

Chondrigen is the principal albuminoid constituent of the matrix of hyaline cartilage.[1] It is an elastic, semi-transparent substance, which is insoluble in hot or cold water and does not swell up materially by treatment either with water or with dilute acetic acid. By prolonged treatment for some hours with water under pressure (at 120°), chondrigen is gradually dissolved with formation of *chondrin*, the solution of which gelatinises on cooling. Hence the hyaline matrix of cartilage appears to bear the same relation to chondrin that the ground-substance of connective tissue bears to gelatin.

For the preparation of chondrin in a state of approximate purity, costal cartilage should be boiled with water for a few minutes, and the perichondrium removed by scraping. It is then cut into fine slices and boiled with water at the ordinary pressure for 24 hours; or preferably heated with water under pressure for 3 to 4 hours to 120°. The solution is filtered while hot—to remove elastin, cellular elements, etc.—and treated with a large excess of alcohol. The precipitated chondrin is washed successively with alcohol and ether, and, if necessary, purified by re-solution in hot water and precipitation by alcohol.

Chondrin so prepared forms a hard, transparent mass, free from taste or odour, and insoluble in cold water. It dissolves in hot water, and the solution gelatinises on cooling, but less strongly than a solution of gelatin of the same strength.

The following is the percentage composition of chondrin according to various authorities:

	Carbon	Hydrogen	Nitrogen	Sulphur	Oxygen
Mulder	49.3	6.6	14.4	0.4	29.3
Fischer and Bödeker	50.0	6.6	14.4	0.4	28.6
Schützenberger and Bourgeois	50.16	6.58	14.18	none	29.08
Von Mering	47.74	6.76	13.87	0.6	31.04

[1] The following results by Hoppe-Seyler show the composition of two typical kinds of human hyaline cartilage:

	Costal cartilage	Articular cartilage
Water	67.67 %	73.59 %
Organic solids	30.13 %	24.87 %
Inorganic solids	2.20 %	1.54 %
	100.00 %	100.00 %

It will be seen from the preceding figures that the elementary analyses of chondrin present considerable discrepancies, and suggest that the substance dealt with is not a definite substance but liable to variations in composition.[1] The results obtained by Morochowetz, and confirmed by Landwehr, Krukenberg, and Mörner, strongly support this view. Morochowetz found that on treating cartilage from various sources with lime- or baryta-water, a 0.5% solution of sodium hydroxide, or a 10% solution of common salt, mucin is dissolved out, and may be thrown down from the solution by acetic acid; while the substance left undissolved is readily convertible by boiling with water into perfectly normal gelatin. According to these observations, chondrin is a mixture of gelatin and mucin, while chondrigen is a mixture of collagen with mucin or hyalogen, the latter component masking its true nature.[2] By micro-chemical examination of the cartilage of the trachea, and the use of staining agents, Mörner detected the existence of a network of collagen, enclosing spherical masses termed "chondrin balls." By treating sections of cartilage with very dilute hydrochloric acid (0.1 to 0.2%), and then with very dilute solution of potassium hydroxide (0.1%), he succeeded in dissolving out the chondrin balls and leaving the network, which by treatment with dilute acids or superheated water was in great part converted into typical gelatin. The chondrin balls were found to consist of a mixture of free chondroitic acid and a mucin called chondromucoid, which on decomposition yielded protein matter and chondroitic acid.

Chondroitic acid, or *chondroitin-sulphuric acid*, $C_{28}H_{51}O_{30}N_8S$, has the characters of a hyaline (pp. 190, 194), though Mörner was unable to verify the existence of the corresponding hypothetical hyalogen. Chondroitic acid yields a reducing sugar when boiled with dilute sulphuric acid. It is interesting on account of its low percentage of nitrogen, and from the fact that the sulphur of the molecule is wholly in the form of an ethereal sulphate.

When acted upon by acids, chondroitin-sulphuric acid splits off sulphuric acid, and yields *chondroitin*, a nitrogenous substance, which

[1] The *inorganic solids* of costal cartilage in 100 parts of ash consisted of: K_2SO_4, 26.66: Na_2SO_4, 44.81; NaCl, 6.11; Na_3PO_4, 8.42; $Ca_3(PO_4)_2$, 7.88; and $Mg_3(PO_4)_2$, 4.55 %.

The *organic solids* in the cells are mostly of protein nature. The ground-substance of the matrix, which forms the greater part of the organic material of cartilage, consists substantially of chondrigen. According to P. B. Hawk, the principal solid constituents of the matrix of cartilaginous tissue are chondromucoid, chondroitin-sulphuric acid, chondroalbuminoid and collagen.

[2] *Chondrin is probably an adsorption compound of simpler substances, i. e.*, gelatin and mucin.

is then converted into another nitrogenous substance called *chondrosin*, acetic acid being formed in the process. This latter substance reduces Fehling's solution even more strongly than dextrose (Hawk). Upon hydrolysis chondrosin is split into glucosamine and glucuronic acid.

Cartilage, the precursor of bone, is thus chemically related to mucin; for both are glycoproteins (glucoproteins). Free sulphuric acid has recently been reported in the slime of snails. From an evolutionary standpoint this relation is of importance, as A. P. Mathews ("Physiological Chemistry," 3rd ed., 1920) remarks, for it shows the chemical relationship between mesodermal and ectodermal tissue. Thus the chitin of arthropod shells (*e. g.* of beetles) yields, on hydrolysis, acetic acid and glucosamine, with some sulphuric acid. See also footnote on page 191.

According to Schützenberger and Bourgeois, the products of the decomposition of chondrin by boiling with baryta water differ from those yielded by gelatin under the same treatment in a much larger (three times) production of acetic acid, and in the entire absence of glycocine. The latter of these results, if correct, is difficult to reconcile with Morochowetz's view of the complex nature of chondrin.

The behaviour of chondrin with solvents and reagents corresponds exactly with that of a mixture of gelatin and mucin. Thus:

	Gelatin	Chondrin	Mucin
Solubility	Insoluble in cold water, alcohol, or ether. Soluble in hot water; such solutions set into a jelly when cold.	Insoluble in cold water, alcohol, or ether. Soluble in hot water; such solutions set into a jelly when cold.	Insoluble in cold water, alcohol, or ether. Insoluble in hot water.
Reaction with:			
Acetic acid	No precipitate.	Precipitate; insoluble except in large excess.	Precipitate; insoluble except in large excess.
Mineral acids	No precipitate.	Precipitate; readily soluble in excess.	Precipitate; readily soluble in excess.
Tannic acid	Precipitate.	Precipitate.	No precipitate.
Mercuric chloride	Precipitate.	Precipitate.	No precipitate.
Lead acetate	No precipitate.	Precipitate.	Precipitate.
Alum	No precipitate.	Precipitate.	Precipitate.
Boiling dilute mineral acids.	No reducing substance formed.	1. Syntonin. 2. Substance reducing cupric oxide.	1. Syntonin. 2. Substance reducing cupric oxide.

The reducing substance formed by boiling chondrin with dilute acids has been called chrondriglucose, but it is said to contain nitrogen, and it is doubtful if it is a true sugar. It is probably identical with the reducing substance resulting from the hydrolysis of mucin by acids.

Mucins[1]

Under the general name of mucin are grouped various closely allied substances which give to many animal secretions, such as snail-slime, synovia, saliva, etc., their characteristic viscid or ropy consistency. A mucin is the characteristic principle of *mucus*, the thick, slimy liquid which covers mucous membranes. Mucins are widely distributed in the animal kingdom, in many cases probably playing a part at present very imperfectly understood. Mucins are contained in many of the tissues, especially the umbilical cord, the tendons, and the submaxillary gland. Mucins are especially abundant in the snail, the mantle and foot containing two distinct varieties.

The method used for the preparation of mucins varies with the material treated, but is based on the solubility of mucins in dilute alkaline liquids, and their precipitation therefrom by acetic acid. Precipitation by alcohol, or saturation of the solution by a neutral salt, may also be employed for the preparation of mucins.

When freshly precipitated, the mucins are glutinous substances which mix with water without undergoing real solution. They dissolve in dilute solutions of the alkali hydroxides, in lime water, and in baryta water. On addition of an acid to either of these solutions, the mucin is precipitated. If acetic acid is used, the precipitate is practically insoluble in excess, nor does hydrochloric acid of a strength ranging from 0.1 to 1.0% of real hydrochloric dissolve most varieties of mucin, but solution is generally effected by acid of 5% strength.

In their natural condition, the mucins are soluble in a 10% solution of common salt, but they become insoluble after precipitation by an acid. By saturating their solutions with common salt, ammonium sulphate, and certain other neutral salts, the mucins are completely precipitated, but not coagulated. The mucins are not

[1] The mucins are compound proteins of the glycoprotein class, and are considered with the albuminoids or scleroproteins only for the sake of convenience. P. A. Levene and his co-workers have done much to reveal the structure of these compounds. See "Hexosamines, Their Derivatives, and Mucin and Mucoids," by P. A. Levera, (Monograph No. 18, Rockefeller Inst. for Medical Research, 1922).

coagulated either by boiling with water or by precipitation with alcohol.

The mucins behave like ordinary proteins with Millon's and Adamkiewicz's reagents (Vol. VIII, pp. 649, 650). With the biuret test, mucins yield a rose-red coloration. No reduction occurs on boiling.

Lead acetate completely precipitates mucins from their neutral or faintly alkaline solutions. Alum also precipitates them, but negative reactions are yielded by most other metallic salts (including mercuric chloride); though all mucins do not behave in the same manner with such reagents. The mucins are not precipitated by tannin.

Such varieties of mucin as have been examined in that respect are not dissolved by pepsin-hydrochloric acid, but are digested by alkaline solutions of trypsin.

By prolonged boiling with concentrated sulphuric acid, the mucins yield leucine and tryosine.

By boiling for 20 to 30 minutes with dilute sulphuric acid, mucins are decomposed into a protein substance which is apparently syntonin, and a pseudo-sugar which reduces Fehling's solution on boiling, is optically inactive, does not undergo the alcoholic fermentation, and is said to contain nitrogen. This decomposition shows the mucins to belong to the class of compound proteins.

The following analyses of mucins from different sources have been recorded:

Origin of mucin	Carbon	Hydrogen	Nitrogen	Sulphur	Oxygen	Observer
1. Mucous contents cyst	52.17	7.01	12.64	....	28.18	Scherer.
2. Snails	50.30	6.84	13.62	1.74	27.53	Hammarsten.
3. Snails (*Helix pomatiæ*)	48.94	6.81	8.50	....	35.38	Eichwald.
4. Tendon	48.3	6.44	11.75	0.81	....	Loebisch.
5. Submaxillary gland	52.31	7.22	11.84	....	28.63	Obolensky.
6. Submaxillary gland	48.84	6.80	12.32	0.84	31.20	Hammarsten.

Of these, No. 2 yielded 0.33, and No. 6, 0.35% of ash. Hammarsten has found that the mucin from the foot of the snail is distinct from that present in the mantle.

Hyalogens and Hyalins.—Hyalin is a term originally applied to the leading constituent of the walls of hydatid cysts, but it was subse-

quently extended by Krukenberg to allied substances obtainable from other animal structures. These substances, in their natural condition, are insoluble, and are termed hyalogens, but by the action of alkalies or water under pressure, they are converted into soluble substances which are generically called hyalins. Thus chondrosin is a hyalogen contained in the sponge, *Chondrosia reniformis;* spirographin is obtained from the cartilage and skeletal tissues of the worm, *Spirographis;* and neossin is the chief constituent of the edible bird's nest. The hyalins corresponding to these hyalogens are called respectively chondrosidin, spirographidin, and neossidin. Similar hyalogens have been described as existing in the vitreous humour of the eye and in hyaline-cartilage (page 190). They agree in their leading characters with the mucins, of which they appear to be simply varieties.

ADDENDUM

ESTIMATION OF DEXTRIN IN THE PRESENCE OF GLUE

Admixture of dextrin powder with glue may be readily detected by microscopic examination, mineral oil or some alcohol of high b.p. being used as a mounting medium. However, if the dextrin has been dissolved in the glue liquor prior to the drying of the glue, the addition is not so obvious. There are some dextrins which will not respond to the iodine test, especially if the glue is highly colored; but any dextrin may with certainty be detected by hydrolysing the mixed glue with dilute hydrochloric acid, and then testing the liquid for dextrose with Fehling's or Benedict's qualitative solution. The protective colloid action of the protein and its hydrolysis products tends to make the copper oxide precipitate so fine that it appears yellow, and, if all the copper is not reduced, the solution may appear to be green.

For the precise estimation of dextrin in glue, the following method proved satisfactory: Soften 0.6 grm. of the glue under examination by soaking it in 10 c.c. of dilute HCl (5 parts of conc. HCl to 100 parts of water). Hydrolyse the mixture slowly at a low temperature (overnight at about 60°). After neutralisation, the solution is made up to exactly 50 c.c., and the dextrose content is determined by Benedict's *quantitative* method.

The following details regarding the preparation and use of Benedict's quantitative solution, are epitomised from S. W. Cole's "*Practical Physiological Chemistry,*" 6th ed. (1920).

Sodium citrate, 200 grm.; sodium carbonate., cryst., 200 grm. (equivalent to 75 grm. of anhydrous sodium carbonate); and 125 grm. of potassium thiocyanate (sulphocyanide); are all dissolved in enough hot distilled water to make up to about 800 c.c. Eighteen grm. of purest copper sulphate crystals (air dried) are dissolved in about 100 c.c. of distilled water, and the solution is poured into the first solution with constant stirring. Five c.c. of a 5 per cent. solution of potassium ferrocyanide are added, and the solution is then made up to 1,000 c.c. with distilled water. The solution is stable, and 25 c.c. are reduced by 0.05 grm. of dextrose.

For the analysis, a 150-c.c. flask is held in a stand over a Bunsen burner, at such a height that the reagent solution can be kept briskly boiling by a *small*

flame. In this flask are placed 3 to 4 grm. of anhydrous sodium carbonate and 25 c.c. of the reagent, and the mixture is heated until most of the carbonate is in solution. The glue solution is now run in *slowly*, until a chalk-white precipitate is formed and the blue colour lessens perceptibly in intensity. The glue solution is then run in still more cautiously, *with constant boiling*, until the disappearance of the last trace of blue indicates the end-point. If less than 5 c.c. of glue solution have been used, the solution should be diluted accurately so that about 10 c.c. will be necessary, and the titration repeated with this diluted solution. Should the mixture in the flask become too thick, boiled distilled water may be added to replace that lost by evaporation.

In using this method, no trace of reduction was shown by a glue known to be pure, which was run as a check or blank in the quantitative test, after having been proved free from reducing substances by the qualitative test. Mixtures of this glue with several commercial dextrins gave concordant results. In converting the dextrose figure into dextrin it should be remembered that commercial dextrins contain variable percentages of water, dextrose, etc. The dextrose figure, multiplied by the factor 0.9, gives a close approximation to the amount of dextrin present. (See *Allen's Commercial Organic Analysis*, 5th ed., (1923), Vol. I, p. 530.)

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STRUCTURAL PROTEINS

BY R. H. MARRIOTT, M.Sc., A.I.C.

The characteristic of the proteins which can suitably be classed as structural substances is their resistance to reagents such as boiling water and dilute acids. Alkalies, on the other hand, digest them more or less readily, especially if warmed. The typical proteins of this class are elastin, reticulin and the fibroin of silk (wild and cultivated) and spider's web.

Elastin.—This protein constitutes the so-called yellow elastic fibres, such as are found in connective tissue and tendons, *e. g.*, skin, where they are interwoven throughout the collagenous or white fibrous tissue enveloping the hair follicles, the nerves and blood-vessels, and in the ligamentum nuchae of herbivorous animals. These fibres are resistant to boiling water and dilute acids, but are slowly attacked by alkalies, especially suspensions of lime (F. L. Seymour-Jones, *J. Ind. Eng. Chem.*, 1922, **14**, 130; J. A. Wilson and G. Daub, *ibid.*, 1924, **16**, 602). It is, however, rather a moot point how far elastin itself is resistant to these reagents, and it would appear not unlikely that the resistance of the fibres is due to an outer sheath of some other material; disintegration of the fibres only occurring when this sheath is ruptured. Evidence of this can be observed when the fibres are digested with trypsin in slightly alkaline solution; the fibres do not become thinner, but break up into globules held in position by this outer sheath.

Both pepsin and trypsin are able to break down elastic fibres, but it would appear that elastic fibres from different sources vary in the ease with which they are digested, those present in calf skin being more resistant to the action of trypsin than are those in goat skin (Marriott, *J. Soc. Leather Trades' Chem.*, 1921, **5**, 280). Strong mineral acids also hydrolyse elastin, yielding the mixtures, on p. 198, of amino acids (E. Abderhalden, *Lehrbuch der physiol. Chem.*, 1909).

Elastin contains only a very small quantity of tyrosine, and since it does not give a black precipitate after heating with concentrated

Glycine	Alanine	Valine	Leucine	Glutamic acid	Phenylalanine	Tyrosine	Proline	Arginine
25.8	6.6	1.0	21.4	0.8	3.9	0.3	1.7	0.3

sodium hydroxide and adding lead acetate, it contains no cystine (Engel, *Z. f. Biol.*, 1897, **27**, 274).

Elastic fibres can be separated from the other proteins in skin by boiling the skin under a reflux condenser with 5% acetic acid until the collagen fibres have dissolved. The residue may be freed from keratin (hairs, etc.) by treatment in the cold with a 5% solution of sodium sulphide, washing well in water and dehydrating with alcohol before removing any traces of grease with ether. Obtained in this way, the elastic fibres form a network which has considerable strength. They are, however, contaminated with reticular tissue. The usual source of elastin is the ligamentum nuchae, but it is by no means certain that the elastin from skin and elastin from tendons are identical. To prepare elastin from the ligaments, the general procedure is to cut up the ligament into thin slices and boil with water for several days. It is then heated to 100° with a 1% solution of potassium hydroxide for some hours and again treated with boiling water. The other proteins are then removed by boiling with 10% acetic acid, and the residue, after treatment with 5% hydrochloric acid, is washed with water, boiled with alcohol and finally with ether.

The material so obtained is yellowish white in appearance and possesses, when moist, a certain amount of elasticity. The following analyses of elastin have been published.

	C	H	N	S	O	Ash	
Ligamentum nuchae....	54.24	7.27	16.70	0.30	21.79	0.90	Chittenden & Hart (*Z. Biol.* 1889, 25)
	55.45	7.41	16.19	....	20.89	....	Müller
Aorta........	53.95	7.03	16.67	0.38	21.97	0.72	Schwarz.

In histological work, elastic fibres are identified by means of Weigert's Resorcinol-fuchsin stain (Lee, *Microtomist's Vade Mecum*)

which colours the elastin fibres a dark blue, leaving the other structures more or less uncoloured.[1] An alternative stain is a 1% solution of gentian violet in water, which gives good differentiation if the material is washed for a prolonged time in water after staining.

Reticulin.—This substance is found as a type of connective tissue, the reticular tissue, of animals. It forms a fine network of fibrils which serve to support the other tissues of the body, such as the white or collagenous fibres and epithelial elements. It behaves very similarly to collagen in relation to the ordinary histological stains, but differs from collagen in its remarkable resistance to chemical reagents. Like elastic fibres, reticular fibrils occur in the form of anastomosing fibrils. Reticulin was originally described by Siegfried in 1892 (see also Young, *J. Physiol.*, 1892, and Tebb, *ibid*, 1902). The presence of this tissue in skin was noted by Jordan Lloyd and Kaye (*Proc. Roy. Soc.*, 1924, **96**, *B*, 293).

The tissue can be isolated from fresh skin by removal of the hair by means of a suspension of lime and then digesting the elastin at 37° for 7 days by means of trypsin at *p*H 8 in the presence of a little toluene. The skin is then boiled in two changes of 0.05 *N* NaOH for periods of four hours. The residue, after washing with warm water, can be dehydrated and degreased with acetone.

Reticular tissue thus prepared gives a slightly positive Million and positive biuret and xanthoproteic reactions. It is slowly broken down by 40% sodium hydroxide in the cold, but rapidly at boiling temperature. *N* NaOH appears, however, to have a more rapid action. It is soluble in concentrated hydrochloric acid but dilute solutions appear to be without effect. Trypsin slowly breaks down the tissue but more rapidly if bacteria also are present. Since reticular tissue *in situ* resists tryptic digestion, it would appear that the method of preparation alters it in some way.

Animal Fibres and Their Recognition

Silk.—Silk is the filament which the silkworm spins and winds round itself before assuming the condition of a chrysalis.

Many varieties of silkworms exist and the silks they produce are of very different quality and commercial utility. The silkworm which lives principally upon the leaves of the white mulberry tree (*Moria*

[1] Some preparations of this stain produce a purple rather than a blue colour and are not quite so specific as those which stain the fibres dark blue.

alba), known as *Bombyx mori*, is the chief producer of silk; but much attention has been given to other silk-producing larvæ, and especially to the Tussah worm, *Antheræa mylitta*, and *A. pernyi*, which feeds on the leaves of the oak, etc. (see *J. Soc. Dyers & Col.*, 1888, **4**, 155).

The *Bombyx* worm secretes a thread from twin orifices situated near the head,[1] and communicating with sacs running one on each side the whole length of the body. The glutinous secretion rapidly coagulates on its exit into the air. As it flows from two distinct apertures, the thread produced is double, the twin fibres being enclosed in a thin, perfectly smooth case, known as "silk-glue," "silk-gelatin," "silk-gum," or "bast" (in France, *grès*). This gelatin is readily soluble in hot soap solution, dilute caustic alkalies, and weak chromic acid, the application of these reagents effecting a separation of the enclosed twin threads. The internal fibre, freed from the gelatinous covering, consists essentially of *fibroin*, while the coating is composed substantially of *sericin*. According to Bolley, in the silk-producing and secreting gland of the worm, glutinous semi-fluid fibroin occurs without admixture with sericin, the latter compound being a product of a subsequent aerial oxidation of fibroin at the moment of formation of the fibre.

Raw commercial silk from the mulberry silkworm is generally regarded as containing about 11% of moisture, 66% of fibroin, 22% of sericin, and 1% of mineral and colouring matter.[2] The mineral matters are chiefly potash, lime, magnesia, and phosphoric acid, the ash containing about 10% of the last-named constituent. The greater part of the mineral matters of raw silk may be removed together with the sericin by prolonged boiling with soap-solution. The residual fibroin retains only about 0.6% of mineral matter.[3]

Silbermann (*Chem. Ztg.*, 1895, **19**, 1683) gives the following analyses of typical types of *mulberry silk:*

[1] Chappe triturated the contents of the glandular organs of silkworms with about one-third their weight of water, and was thus able to blow permanent globes and diversely shaped vessels with them.

[2] The colouring matter of yellow silk has been investigated by Dubois (*Compt. rend.*, 1890, **111**, 482), who has isolated from it several substances resembling in many respects vegetable carrotin. These are yellowish-red and crystallisable, altered by exposure to light and air, soluble in alcohol, ether, chloroform, and benzene to golden-yellow solutions, and in carbon disulphide to a brownish-red solution. They give absorption spectra free from bands, and dissolve in strong sulphuric acid, with blue colouration changing to green, destroyed by addition of water. In addition to these colouring matters, Dubois isolated a deep greenish blue pigment, which is present in very small quantity.

[3] Carter Bell (*J. Soc. Chem. Ind.*, 1897, **16**, 304) states that the ash of a genuine silk varies according to the district in which it is produced, and averages 0.5%. He found 0.35% of ash in a pure white silk, while a Chinese salmon-coloured silk contained only 0.25%. In the raw state, the ash of silk varies very much. In good Italian silk it is about 1%. Yama-mai silk sometimes yields as much as 8% of ash, which is greatly reduced by successive treatments of the fibre with alcohol, dilute sulphuric acid, and boiling soap solutions.

Kind of silk	Fibroin	Ash of fibroin	Sericin	Wax and fat	Salts
White; cocoons.......	73.59	0.09	22.28	3.02	1.06
White; raw	76.20	0.09	22.01	1.36	0.30
Yellow; cocoons......	70 02	0.16	24.29	3.46	1.92
Yellow; raw.........	72.35	0.16	23.13	2.75	1.60

A sample of *mulberry silk* was found by Richardson to contain:

	Air-dry	Dried at 100°
	%	%
Water................................	12.50	....
Fatty substances.....................	0 14	0.16
Resinous substances	0.56	0 64
Sericin..............................	22.58	25.81
Fibroin..............................	63.10	72.11
Mineral matter	1.12	1.28
	100.00	100.00

Raw tussah silk was analysed by Bastow and Appleyard (*J. Soc. Dyers & Col.*, *1888*, **4**, *88*) with the following results, the sample being reeled and previously dried at 100°.

Soluble in hot water................................	21.33%
Subsequently dissolved by alcohol (chiefly fatty acid)...	0.91%
Subsequently dissolved by ether	0.08%
Total loss by boiling off with 1% solution of curd soap..	26.49%
Mineral matters.....................................	5.34[1]%

Inoye and Hirasawa, *J. Tokyo Chem. Soc.*, *1918*, **39**, 300; *1920*, **41**, 876) examined the cocoon silk of different species of silk worm and found the amino acids (using the ester method).

The cultivated silk contained 24.2% of sericin.

Silk appears to contain tryptophane, and according to Hiratsuka (*Biochem. Z.*, 1925, **157**, 46), wild silk contains more of this amino acid than does the cultivated variety.

[1] The *ash* of tussah silk was found by Bastow and Appleyard to have the following composition: K_2O, 31.68; Na_2O, 12.45; CaO, 13.32; MgO, 2.56; Al_2O_3, 1.46; SiO_2, 9.79; P_2O_5, 6.90; SO_3 8.16; CO_2, 11.14; Cl, 2.89; less O equivalent to Cl, 0.65; total 99.70. As sulphur is not a constituent of the organic compounds of mulberry silk, the occurrence of sulphates in the ash of tussah silk is characteristic. The presence of a notable proportion of alumina is to be noted, as aluminium is not a generally recognised constituent of animal products, and is contained in so few plants that its presence in the secretion of the silkworm is difficult to account for. It is probable that the greater part of the ash found by Bastow and Appleyard in Tussah silk was derived from adhering impurities. The fibroin prepared from the same silk contained only 0.226% of ash.

	Cultivated	Tussah	
		(Japan)	(China)
Glycine	25.42	12.38	16.80
Alanine	22.58	15 27	11 87
Leucine	0.62	0.27	0.25
Aspartic acid	0.11	2.37	4.08
Glutamic acid	trace	trace	trace
Proline	0.32	0.26	0.23
Serine	2.77	0 55	0.47
Phenylalanine	0.25	0 37	1.18
Tyrosine	5.68	6.62	3.86

When silk is examined by means of the X-ray spectrograph it shows evidence of crystal structure. Brill (*Annalen*, 1923, **434**, 204), suggests that it is composed of at least two proteins, one of which is crystalline.

Silk Conditioning.—In a damp atmosphere, raw silk will take up a large proportion of moisture (25 to 30%) without any indication of the fact, other than increase in weight. To ascertain the proportion of moisture in raw silk, a number of hanks of the sample are dried at 105° in a current of air until constant in weight. To the weight thus obtained it is usual to add 11%, the result being regarded as the weight of the silk in a normal condition of humidity.

This per cent. is recognised as a trade standard, and the different Conditioning Houses in the silk districts are established for testing the same.

Silk Scouring.—In the process of scouring silk, the silk-gum or sericin is removed more or less completely. This is generally effected by heating the hanks of raw silk in a solution of soap at 90 to 95°, the process being sometimes repeated with a fresh soap-bath. The fibre at first swells up, but as the gum dissolves, it gradually acquires a soft and silky feel. The resulting liquid, known as "boiled-off liquor", is a useful addition to the dye-bath in dyeing silk with coal-tar colours. The last traces of sericin are removed by washing the silk in water at 60°, to which some soap and sodium carbonate have been added. The silk is sometimes again boiled with soap solution, rinsed with warm sodium carbonate solution, and lastly washed with cold water. If the silk is to remain undyed or to be dyed a pale colour, it is sometimes exposed while moist to sulphur dioxide

gas for 5 or 6 hours, or to the action of hydrogen peroxide. The former operation, which has for its object the bleaching of the silk, is, in many cases, repeated several times.

Degumming has been carried on at lower temperatures in more strongly acting alkaline solutions (NaOH, etc.) in the presence of a protective substance, such as dextrose.

Besides the above process, which is employed to produce completely ungummed silk, involving a loss of 22 to 33% of the original silk, *souple silk* is produced when only from 8 to 12% of sericin is removed;[1] and when *écru* silk is required, the hanks are simply washed with hot water containing a little soap, whereby a loss of only 3 to 4% results.

Sericin, obtained by treating silk with water with or without pressure, is a yellowish, transparent substance, resembling gelatin. It dissolves in hot water, hot soap solutions, and dilute alkali hydroxide. The hot aqueous solution forms a strong jelly on cooling. Aqueous solutions of sericin are precipitated by alcohol, tannin, basic lead acetate, stannous chloride, chlorine, and bromine. Potassium ferrocyanide in presence of acetic acid gives a greenish precipitate.

Skelton and Johnson (*J. Amer. Chem. Soc.*, 1925, **47**, 412) employed the following method for separating the sericin. The silk was autoclaved with water for half an hour at 5 lbs. pressure, and to each 100 c.c. of liquor, 15 grm. of solid ammonium sulphate were added. A gelatinous precipitate was obtained which was insoluble in cold water. (This substance did not support bacterial growth.) A further precipitate was obtained by saturating the filtrate with ammonium sulphate. These workers consider that sericin is a mixture of two proteins.

By the action of dilute sulphuric acid on sericin, Crämer obtained 5% of tyrosine (distinction from gelatin) and 10% of serine, a substance having the constitution of an amino-glyceric acid, $CH_2(OH).CH(NH_2).COOH$.[2]

[1] Soupling seems to alter the nature of the silk-gum as well as reduce its percentage in the raw silk fibre, whereby the sericin is rendered soft, pliant, and lustrous. Souple silk is stated by Silbermann to possess the property of taking up vegetable and mineral weighing materials in a greater degree than either raw or boiled-off silk. For coloured silks, the soupling is often carried out in a boiling bath of cream of tartar or acid sodium sulphate. For black silk, the soupling may take place in the weighting and dyeing processes as tannic acid acts in this direction at a temperature above 68–70°.

[2] Serine seemingly resembles glycocoll (Vol. VIII) in its power of uniting with both bases and acids. It differs from alanine or amino-propionic acid by an atom of oxygen. By the action of nitrous acid, alanine yields lactic acid, while serine gives glyceric acid when similarly treated.

According to Mülder, sericin has the following empirical formula and ultimate composition. For convenience of comparison, the formula and composition of fibroin (according to Mülder and Crämer) are also given:

	Sericin, $C_{15}H_{25}O_8N_5$	Fibroin, $C_{15}H_{23}O_6N_5$	Fibroin (Crämer)
Carbon	42.60	48.80	48.60
Hydrogen	5.90	6.23	6.40
Nitrogen	16.50	19.00	18.89
Oxygen	35.00	25.00	26.11
	100.00	100.00	100.00

Bolley attributed to sericin the composition: Carbon, 44.32; hydrogen, 6.18; nitrogen, 18.30; and oxygen, 31.20%.

An excess of oxygen in sericin over that in fibroin is possibly due to the greater oxidation of the surface of the fibre, the conversion of fibroin into sericin being assumed to take place by reaction with water and oxygen:

$$\underset{\text{Fibroin.}}{C_{15}H_{23}O_6N_5} + H_2O + O = \underset{\text{Sericin.}}{C_{15}H_{25}O_8N_5}$$

According to Inoye and Hirasawa (*loc. cit.*) cocoon silk may contain 24.2% of sericin and this on hydrolysis yields glycine 3.86%, alanine 3.47%, leucine 0.39%, aspartic acid 3.91%, glutamic acid 0.81%, proline 0.35%, serine 5.89%, phenylalanine 0.49% and tyrosine 3.18%. Fischer and Skita (*Z. physiol. Chem.*, 1902, **35**, 221) state, that sericin gives, on hydrolysis, diamino-acids including arginine and the same monoamino-acids as fibroin. Türk (*Z. physiol. Chem.*, 1920, **111**, 69) obtained the following figures: leucine 1.69%, serine 6.81%, tyrosine 5.69%, arginine 4.56%, lysine 1.69% and histidine 1.02%.

Fibroin is related in character to elastin (page 197), and is obtained by treating silk successively with boiling water, alcohol, ether, and acetic acid. The resultant fibroin retains the fibrous character of the original silk, but is generally white, of good lustre, and soft to the

$CH_3.CH(NH_2).CO_2H$ Alanine.	$CH_3.CH(OH).CO_2H$ Lactic acid.
$OH.CH_2.CH(NH_2).CO_2H$ Serine.	$OH.CH_2.CH(OH).CO_2H$ Glyceric acid.

touch.[1] On ignition, fibroin evolves an odour resembling that of burnt horn. Fibroin does not dissolve in ammonia or solutions of the carbonates of alkali-metals, and is not appreciably affected by 1% solution of potassium or sodium hydroxide, but is dissolved by stronger lye, especially at high temperature. It is, however, never treated with such solutions when the lustre of the silk is a consideration. On dilution, a precipitate is obtained which is said to consist of unaltered fibroin, but this is very doubtful. Fibroin is soluble in hot glacial acetic acid, and also in strong hydrochloric, nitric, sulphuric, or phosphoric acid. It also dissolves in alkaline solutions of nickel, zinc, or copper salts, etc.[2]

The proportion of fibroin in the silk from *Bombyx mori* has been variously stated according to the method employed for its determination. Thus Mülder, who prepared it by boiling the raw silk with acetic acid, gives it as 53 to 54%. Städeler, by acting on the silk with a 5% solution of sodium hydroxide in the cold, obtained from 42 to 50% of fibroin. Crämer, by heating the silk with water at 133°, obtained 66%; and Francézon, by boiling the silk twice with soap, and then treating it with acetic acid, found 75% of fibroin. Vignon, as the result of an improved method of determination, states the average proportion of fibroin in raw silk at 75%. This agrees with the results obtained in practice. These higher figures doubtless refer to silk previously dried at 100°.

Weyl (*Ber.*, 1888, **21**, 1529) found that by boiling purified white silk under a reflex condenser for 18 hours with dilute sulphuric acid (1 : 5), it dissolved to a yellowish-brown liquid, leaving a few globules of a fatty acid. From the product Weyl isolated 5.2% of tyrosine, 7.5% of glycocoll, and 15% of a crystalline sublimable compound which was apparently α-alanine. The remaining products (72%) could not be isolated or identified.

[1] For the preparation of pure fibroin, Vignon (*Compt. rend.*, 1892, 115, 17, 613) recommends that a skein of raw white silk weighing about 10 grm. should be boiled for 30 minutes in 1,500 c.c. of water containing 15 grm. of neutral soap. The silk is then rinsed successively in hot and in tepid water to remove the soap, hydro-extracted, and submitted to a repetition of this treatment, a fresh soap-bath being used in which the silk is immersed for 20 minutes. The silk is then rinsed with water, passed through dilute hydrochloric acid, again rinsed with water, and then washed twice with 90% alcohol. The fibroin thus obtained is brilliant, white, and soft, and leaves only 0.01% of ash on ignition. It was found by Vignon to have the elementary composition: C, 48.3; H, 6.5; N, 19.2 and O, 26.0%.

[2] Bastow and Appleyard (*J. Soc. Dyers & Col.*, 1888, **4**, 89) have pointed out that owing to the fact that the fibroin of tussah silk is acted on by solvents far less readily than the fibroin of mulberry silk, the two products are probably chemically distinct. Tussah fibroin was prepared by repeatedly boiling the silk with a 1% solution of curd soap, washing with water, extracting with hydrochloric acid, again washing with water, drying, and extracting successively with alcohol and ether. Thus purified, tussah fibroin had the following elementary composition (exclusive of 0.226% of ash): Carbon, 47.18; hydrogen, 6.30; nitrogen, 16.85; and oxygen, 29.67%.

The constitution of silk from a chemical standpoint has been considered in detail by Silbermann (*Die Seide,* Band II, 1897). (See also H. Silbermann, *Chem. Ztg.*, 1903, **17**, 1693), and the chemistry of tussah by Wardle (*Textile Manuf.*, 1891, p. 563).

The decomposition products of fibroin are given by Fischer and Skita (*Chem. Ztg. Rep.*, 1901, **25**, 274) as follows (per 100 pt.): 10 of *l*-tyrosine, 21 of *d*-alanine (aminopropionic acid), 1.5 *l*-leucine, 1.5 *l*-phenylalanine, 36 of glycine, traces of other amino-acids and a trace of certain diamino-acids. Abderhalden (*Z. physiol. Chem.*, 1922, **120**, 207) gives the following figures, *d.* alanine 25.0, *l.* leucine 2.5, phenylalanine 1.5, *l.* serine 1.8 and *l.* proline 1.0.

Silk-fibroin resembles proteins in its behaviour with Millon's and Adamkiewicz' reagents and with the biuret test.

Richardson found combed and well-purified mulberry-silk to absorb 30% of iodine when treated with Hübl's reagent (Vol. II). The product retained a deep yellow colour, even after washing with potassium iodide and water. Attempts to acetylate fibroin gave entirely negative results.

Silk is acted on by dilute nitric acid. When immersed for about a minute in nitric acid of 1.133 sp. gr., at a temperature of about 45°, and then washed thoroughly with water, silk acquires a characteristic yellow colour, fast to light and air. Pure nitric acid (free from nitrous compounds) does not produce the colour, which varies in intensity with the proportion of nitrous compounds present. Nitrous acid, produced by adding hydrochloric acid to a solution of sodium nitrite, dyes silk a pale-yellow colour which is affected by light; but if the silk thus treated be then immersed in dilute nitric acid or in a mixture of hydrochloric acid and potassium permanganate, the yellow colour becomes deeper and permanent to air and light. If this colour be treated with an alkali, the yellow shade is considerably deepened, the shade obtained varying with the base used, being lightest with ammonia and approaching red with baryta Alkaline carbonates produce the same intensity of colour as the corresponding hydroxides. It is said the alkali cannot be removed by washing. Nitro-silk is slowly decolorised when boiled in a concentrated acid solution of stannous chloride, but alkaline reducing agents appear to have no action. Nitro-silk behaves like ordinary silk with solvents, except that when treated with concentrated sulphuric acid it swells up and gives a sticky mass similar to tha

given by ordinary egg-albumin. Vignon and Sisley, to whom the above observations on this product are due (*Compt. rend.*, 1891, **113**, 802), analysed two samples of white Canton silk, which had been degummed and purified by successive treatments with boiling soap solution, distilled water, hydrochloric acid, water and alcohol. One of the samples thus purified was "dyed" with nitrous nitric acid, by which treatment it gained 2% in weight. The following figures show the composition of the dyed and undyed fibroin:

	Undyed silk	Dyed silk
Carbon	48.3%	46.8%
Hydrogen	6.5%	6.5%
Nitrogen	19.2%	21.6%
Oxygen	26.0%	25.1%
	100.0%	100.0%

Action of Solvents on Silk.—Silk is gradually dissolved by cold concentrated sulphuric acid to form a slightly brownish-coloured solution.[1] Hydrochloric acid dissolves it without additional colour to a limpid liquid. Zinc chloride and alkaline solutions of copper and nickel also dissolve silk. Cold concentrated solution of potassium or sodium hydroxide dissolve silk completely, but 2% solutions dissolve the sericin only, leaving the fibroin. The solutions are not precipitated by dilution with water, but evolve ammonia when heated.

Different varieties of silk differ materially in their behaviour with reagents. Von Höhnel claimed to have effected a quantitative separation of a mixture of real silk, Yama-mai silk, sheep's wool, and cotton into its four constituents, by boiling the mixture with hydrochloric acid for 30 seconds whereby the real silk only was dissolved; while on continuing the boiling for 2 minutes with concentrated hydrochloric acid, the Yama-mai silk passed into solution. On heating the residue with potassium hydroxide solution, the wool was dissolved, while the cotton remained unchanged. The analysis of mixed fibres is described in detail on page 217 *et seq.* Bastow and Appleyard (*J. Soc. Dyers & Col.*, 1888, **4**, 89) observed the following differences between mulberry and tussah silk:

[1] By diluting the solution of silk in cold concentrated sulphuric acid with water, boiling, neutralising with milk of lime, and evaporating the filtered solution to dryness, Richardson obtained 15% of glycine, leucine, and tyrosine, and 85% of a substance having the odour and appearance of glue, readily soluble in water, but insoluble in alcohol.

Treated with	Mulberry silk (*Bombyx mori*)	Tussah silk (*Antheræa mylitta*)
A hot 10% solution of sodium hydroxide.	Completely dissolved in 12 minutes.	Required 50 minutes for complete solution.
Cold concentrated hydrochloric acid (sp. gr. 1.16).	Dissolved almost instantly.	Only partially dissolved in 48 hours.
Cold concentrated nitric acid.	Dissolved in 5 minutes with light-yellow colour.	Dissolved in 10 minutes to a light-brown solution.
Neutral solution of zinc chloride of 1.725 sp. gr.[1]	*Dissolved almost instantly.*	*Required considerable time* for complete solution.
Saturated aqueous solution of chromic acid mixed with an equal measure of water.	*Dissolved immediately.*	*Slowly acted on.*

It must be remembered, however, that mulberry silk will dissolve in 10% or even 5% sodium hydroxide solution in a much shorter period (2–3 minutes).

F. Filsinger[1] (*Chem. Ztg.*, 1896, **20,** 324) has shown that mulberry silk is readily soluble in an alkaline solution of copper hydroxide in glycerin, whereas Tussah silk is scarcely affected by this reagent.

Examination of Raw Silk.—In the analysis of raw silks the following results obtained by Gianoli (*Rev. Gen. d. Mat. Col.*, 1906, **10,** 199) are of interest. On the average the matter soluble in 3% boiling soap solution was from 21.44–25.9. In water at 50–55°, 0.44–1.05 was extracted; in ether from 0.1–0.45%.

This strength of soap solution is in excess of that actually required. In the actual assay of raw silk, for the amount of silk gum present, a 1% soap solution is quite satisfactory. This may be followed by a 0.2% solution *if it is thought necessary.*

Gnehm (*Farb. Zeit.*, 1903, **14,** 69) has recorded adulteration of raw silk with fat up to 8.5%. It must, however, be remembered that raw silk is very often treated with a strong solution of fatty matters in order that it may spin and weave more easily, but in the case mentioned it seems that the imported raw silk in the original skeins contained the fat.

Optical Activity.—Solutions of silk are lævorotatory. L. Vignon (*Compt. rend.*, 1891, **113,** 802; 1892, **114,** 129) removed the colouring matter from silk (from *Bombyx mori*) by repeated treatment with hot alcohol acidified with hydrochloric acid. The silk thus purified was immersed in a cold 3% aqueous solution of sodium hydroxide, when a yellowish, limpid solution of *sericin* was obtained having an

[1] Persoz also states that mulberry silk is almost immediately (in less than 30 seconds) dissolved by a boiling solution of zinc chloride of 1.45 sp. gr., while tussah silk is only very slowly acted on; and a rough separation of the two silks may be effected by immersing the mixture in the hot reagent for 1 minute, and then removing and washing it, when the residue will consist of tussah silk only.

optical activity of $[\alpha]_D = -39.2°$. Silk from the same source was boiled twice with a 10% solution of soap, which was washed away by water after each boiling, the residual silk washed with acidulated water (0.1% of hydrochloric acid), and finally with alcohol. The white *fibroin* thus obtained was dried and dissolved in moderately strong hydrochloric acid, when a clear viscous solution was obtained having after dilution an optical activity corresponding to $[\alpha]_D = -40°$, which was materially altered by further dilution, or by the addition of excess of ammonia. Solutions obtained by Vignon in a similar manner from other varieties of silk showed a value for $[\alpha]_D$ from $-9°$ to $-43.6°$, mean $-29.6°$, for the alkaline solution of the sericin; and from $-39.4°$ to $-50°$, with a mean of $-44°$, for the hydrochloric-acid solution of the fibroin. The fibroin of *Yama-mai* was found to be insoluble in hydrochloric acid, but soluble after some time in cold concentrated sulphuric acid.

Microscopical Characters.—The silk from *Bombyx mori* (mulberry silkworm) and *Saturnia spina* is a homogeneous, hyaline, formless fibroin thread resembling glass-rod, and rarely exhibits signs of striation. The fibre of real silk is distinguished from its substitutes by the microscope. Its fibre is round, and of less diameter than its substitutes, with the exception of Thiele artificial silk.

On the other hand, the fibres of tussah and other "wild" or exotic silks, now much used on account of their low price, consist of bundles of circular threads varying in diameter from 0.0003 to 0.0015 millimetre, (3 to 15μ) which give the main fibre a striated appearance. The fibrillæ are agglutinated by a matter not greatly differing from their own substance, and not capable of being sharply distinguished or easily separated from the fibroin, as the silk-gum of mulberry silk is from the enclosed fibroin. Many of the twin fibres of the exotic silks are flattened to a marked extent. The wild silks are darker in colour; Tussah being brown in the natural state (see Fig. 2).

Filsinger (*Chem. Ztg.*, 1909, **20**, 324) describes the cross-section of Tussah fibres as larger and flatter than that of mulberry silk, and showing many fine air-tubes. Characteristic bands frequently cross the fibres in an oblique direction, giving them a microscopic appearance similar to that of cotton.

Von Höhnel (*Dingl. polyt. J.*, 1885, **146**, 465) has recorded the following characters of raw silk from different sources. The figures

refer to the maximum diameter of single threads and are expressed in thousandths of a millimetre:

Kind and origin of silk	Diameter in μ	Colour and microscopic appearance	
		Broad side	Narrow side
Mulberry silk from *Bombyx mori*	20 to 25.	White or yellowish-white; shining.	White or yellowish-white; shining.
Senegal silk from *Bombyx Faidherbi*.	30 to 35.	Shining yellowish or brownish-white, or pale yellow, grey, brown, and occasionally bluish-white.	Grey, brown, or black, with occasionally lighter shades.
Ailanthus silk from *Bombyx cynthia*.	40 to 50.	Shining yellowish-white, with yellow, brown, brownish-grey spots.	Dirty grey or brown to black, with green, yellow, red, violet, or blue spots.
Yama-mai silk from *Antheræa yamamai* (Japan).	40 to 50.	Bluish-white, with dark blue, blue, and black shades.	Glaring and fine colours with dark or black shades.
Tussah silk from *Actius selene* (?).	50 to 55.	Irregular in thickness. Thickest parts with grey and blue spots; thinner parts, bluish-white, yellow, or orange-red.	Dark grey, with pink or light-green spots.
Tussah silk from *Antheræa mylitta*.[1]	60 to 65.	Similar to *A. selene*, but spots orange-red, red, or brown.	Similar to *A. selene*.

[1] The cocoons of the larvæ of *Antheræa mylitta* are firm and hard, of a silvery drab colour, egg-shaped, and much larger than those of the true silk-worm. The product is used largely for the manufacture of silk plush and buff-coloured Indian silks.

Other wild silks are the *Eria* silk of India, the *Muga* silk of Japan, the *Fagara* or *Atlas* silk of China, etc.

Woodcuts illustrating the magnified appearance of a number of varieties of silk have been published by Wardle (*J. Soc. Dyers & Col.*, 1885, **1**, 196); and details of the tension, strength, and diameter of the fibres have been recorded by the same author (*J. Soc. Arts*, **33**, 671).

The microscopic appearance of tussah silk has been described and illustrated by Bowman (*J. Soc. Dyers & Col.*, 1888, **4**, 90).

Interesting particulars respecting wild silk have been given by C. Grosseteste (*J. Soc. Dyers & Col.*, 1888, **4**, 155).

Silbermann gives the diameter of silk filaments as follows:

Nature of silk	*Average size*	*Limits of size*
Mulberry silk	24μ.	18–26μ.
Indian tussah	45μ.	40–60μ.
China tussah	70μ.	62–80μ.

A long series of papers on the chemical composition and structure of different varieties of silks has been given by Strauch (*Z. physiol. Chem.*, 1910 and 1911). These may be referred to where details are required as to decomposition products, etc.; similar work has been done by Abderhalden (*Z. physiol. Chem.*, 1911, **72**, 1). (Also see

Abderhalden and Schmid, and Abderhalden and Walder, *Z. physiol. Chem.*, 1910, **64**, 460, and Abderhalden, 1910, **65**, 417 and 1910, **66**, 13.)

Particulars concerning the African wild silks are given by Barwick (*Bull. Imp. Inst.*, 1910, **8**, 150).

The gum of Indian tussah silk gave the following yields of mono-amino-acids calculated on the ash-free material dried at 120°: glycine 11.5, alanine 9.8, leucine 4.8, serine 5.4, aspartic acid 2.8, glutamic acid 1.8, phenylalanine 0.3, tyrosine 1.0, proline 3.0%.

Tussah silk is bleached after degumming in "Bensoap" (1% solution) to which 2–5 kilos of sodium perborate to the 1,000 litres has been added, treatment being carried out at 60–70°. It is then decolorised in a slightly alkaline hydrogen peroxide solution (6 volumes) at 30–40°. After treating with oxalic acid the material is bleached with sodium hydrosulphite. (Beltzer, *Rev. Gen. Mat. Col.*, 1911, **15**, 36.)

Physical Examination of Silk in Mixed Fibres.—The foregoing observations refer to the microscopic appearances of different varieties of silk, but the microscope also serves to readily distinguish silk from cotton, wool, and other textile fibres. The illustrations on pp. 808–810 indicate the relative appearance of silk and other fibres under a magnifying power of 80 diameters.

Under the microscope, *silk* appears as a slender, homogeneous, solid cylindrical and continuous filament, free from either scales or medullary substance. Sheep's *wool* appears thicker than silk, and has a perfectly circular stalk with tile-shaped scales. *Linen* (flax) is cylindrical and never flat; it is not stiff or twisted, and is characterised by the narrowness of its medullary tube. *Hemp* resembles linen-fibre, being easily broken; its ends branch out stiffly, and its tube is open. *Cotton* fibres are long, flat, and resemble a twisted band. Mercerised cotton is rounder.

Detection of Animal Fibres by Chemical Means.—Animal fibres, such as silk, wool, and hair, contain nitrogen, and evolve an odour of burnt feathers when ignited. They are dyed by magenta and picric acid without a mordant, and are coloured yellow by nitric acid and red by Millon's reagent. Tussah silk (unbleached) gives a chocolate brown.

The reactions shown on p. 215 distinguish silk qualitatively, and to some extent quantitatively, from other fibres.

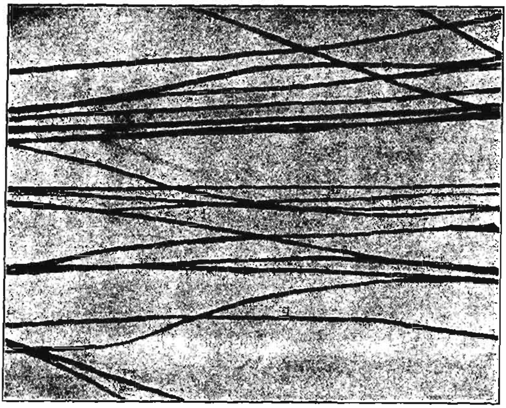

FIG. 1.—Ordinary Silk. × 80. (*Bombyx mori.*)

FIG. 2.—Tussah Silk. × 80. (*Saturnidae.*)

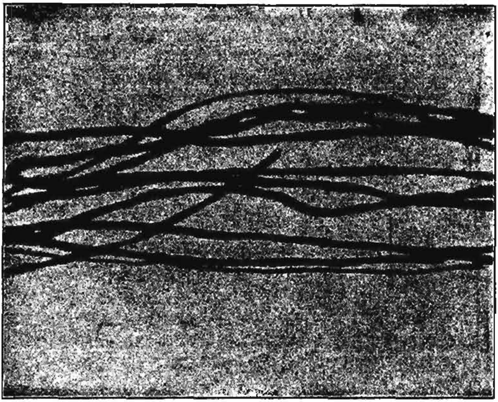

FIG. 3.—Cotton. × 80.

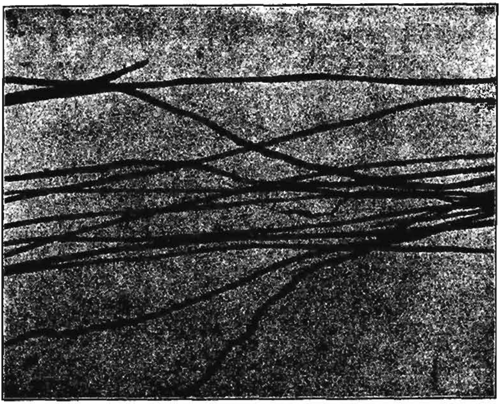

FIG. 4.—Mercerised Cotton. × 80. (*Egyptian.*)

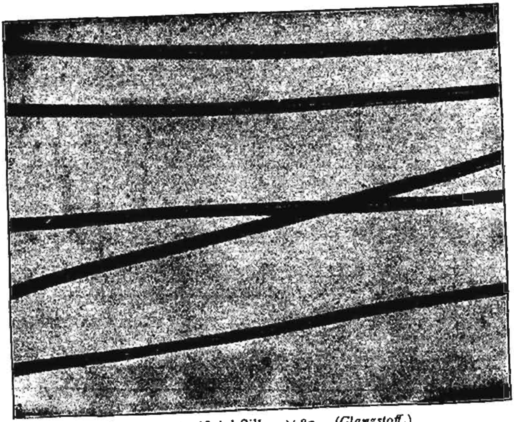

FIG. 5.—Artificial Silk. × 80. (*Glanzstoff.*)

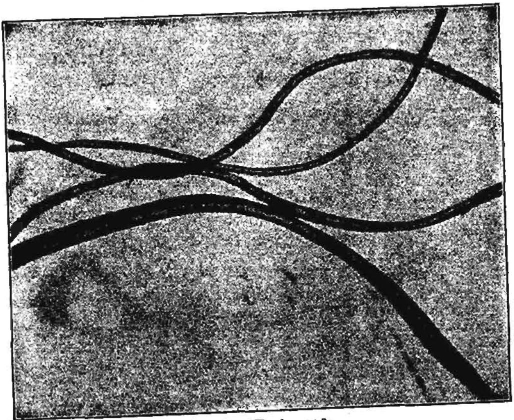

FIG. 6.—Wool. × 80.

	Silk, wool, fur, or hair		Cotton or linen
Heated in small test-tube.	Brittle, carbonaceous residue, with smell of burnt feathers. Condensed moisture is alkaline to litmus.		Charring and smell of burning wood. Condensed moisture is acid to litmus.
Boiled in an aqueous solution of picric acid, and rinsed in water.	Dyed yellow.		Unchanged.
Treated with cold nitric acid of 1.2 sp. gr.	Coloured yellow.		No change of colour.
Boiled with Millon's reagent.	Red coloration.		No change of colour.
Moistened with dilute hydrochloric acid and dried at 100°.	Unchanged.		Powders on friction.
	Silk (mulberry)	Wool, fur, or hair	
Heated to boiling with conc. hydrochloric acid.	Dissolved.	Swells, without at once dissolving.	Mostly undissolved.
Boiled with a concentrated solution of basic zinc chloride.	Dissolved.	Unchanged.	Unchanged.
Treated with a cold concentrated solution of ammoniocupric oxide (Schweitzer's reagent).	Dissolved. Not precipitated from the solution by salts.	Undissolved. Dissolves on heating.	Dissolved slowly. Solution precipitated by salts.
Treated in the cold with 10% caustic soda.	Undissolved.	Dissolved.	Undissolved.
Boiled with 2% sodium hydroxide solution.	Dissolved. Solution not darkened by lead acetate; negative reaction with sodium nitroprusside.	Dissolved. Solution gives black or brown precipitate with lead acetate, and violet colour with sodium nitroprusside.	Unchanged.
Behaviour with Molisch's test.	Dissolved; with little coloration.	Undissolved; yellow or brown coloration.	Dissolved; deep violet colour.

Reactions for distinguishing mulberry silk from tussah silk are given on page 221.

Lecompte's Test Modified.—The fibre or fabric is treated with an acid solution of nitrous acid, prepared by adding excess of hydrochloric acid to a 1% solution of sodium nitrite. The operation is conducted in the dark. After washing, it is introduced into an alkaline solution of β-naphthol, prepared by dissolving that substance in a weak solution of sodium hydroxide to which lead acetate has been added to produce lead plumbite. Wool is coloured black by the interaction of the sulphur in the fibre with the lead. Silk is dyed a deep red, and cotton or artificial silk remains colourless. According to Dreaper the time taken to "diazotise" the fibre must not be less than 30 minutes, and the sodium nitrite solution will act more readily if its strength is increased to 5%. The free acid must be washed out, before the sample is immersed in the β-naphthol and lead acetate

solution. A temperature of 100° is necessary to develop the red shade on the silk to its maximum, when it is of a dull purple colour.[1]

Dreaper's Test.—In order to avoid the difficulties connected with Lecompte's test, the following solution is proposed: A solution containing 2 grm. of acetate of lead, 2 grm. of sodium hydroxide (fused), and 2 grm. of picric acid, or 0.3 grm. of magenta, per 100 c.c. is boiled with the fibre to be examined for two minutes, which is then washed. A silk fibre will be dyed yellow or red respectively; wool will be dark brown or black; cotton will remain colourless. When a thread is thought to contain a mixture of these fibres, it is best examined under the microscope, when the colour test will at once confirm the ordinary microscopical examination. This test may, of course, be made in daylight and only entails the use of one solution. Artificial silks remain white. (*J. Soc. Dyers and Col.*, 1913, **29**, 78.)

Grothe (*Z. anal. Chem.*, 1864, **3**, 153) distinguished silk from wool by the following reactions. The fibre is heated to 130°. Under these conditions wool turns to a golden yellow shade, gives an odour of carbon disulphide and ammonia, and curls up. Silk does not become coloured under 140° and does not curl. When the fibre is moistened with potassium hydroxide, and then dipped into a solution of copper sulphate, and exposed to the air, wool rapidly turns brown through the formation of cupric sulphide, whilst silk or cotton remains colourless. Wool dissolved in sodium hydroxide and neutralised with tartaric acid gives with copper sulphate a dark brown colour, whilst silk gives a fine violet coloration.

Liebermann's Test.—Sodium hydroxide is added to a concentrated solution of magenta until the solution is colourless. After filtering, the fibre is dipped into the liquid, withdrawn and well washed with water. Wool or silk are dyed a red shade, while cotton remains colourless. The colour test is rendered more delicate by immersing the fibre in dilute acetic acid, after washing it in pure water.

Liebermann's reaction and that with picric acid may be employed to render visible the animal fibres in a mixed yarn or fabric. Wool (and hair) fibres may be rendered visible by immersing the material in a dilute boiling solution of sodium hydroxide, to which a little lead acetate has been added. The wool will be turned brown or black

[1] Dreaper varied this test by boiling the sample in the nitrous acid solution for two minutes in ordinary light instead of the longer process in the dark (*J. Soc. Dyers & Col.*, 1913, **29**, 78).

from the formation of lead sulphide, whilst silk and vegetable fibres are unaffected.

In mixtures of silk and wool Dreaper finds that treatment with concentrated sulphuric acid under the microscope may give valuable information. A thread is placed on a micro slide and covered with an ordinary circular cover. While the sample is being examined a drop of concentrated sulphuric acid is dropped onto the outer edge of the cover. By capillary action it follows the line of the fibre, and any silk present is dissolved within 2 minutes, leaving the wool intact. The actual process of solution may be observed.

The foregoing tests are of value chiefly for the preliminary examination of undyed fibres or after dyes have been removed.

Quantitative Estimation of Silk

Various methods of *determining the proportion of silk* in mixed fabrics have been proposed, but most of them are imperfect, and the best are not free from errors, owing to the absence of any reagent which will dissolve either cotton, silk, or wool, without at the same time more or less attacking the other two fibres.

Silk has been estimated in plush by boiling the fabric with a 2% solution of sodium hydroxide, but this reagent acts to a serious extent on cellulose, especially in presence of air. A reagent sometimes recommended for the solution of silk is prepared by dissolving 16 grm. of copper sulphate in 140 to 160 c.c. of water, and adding from 8 to 10 grm. of pure glycerol. A solution of sodium hydroxide is then gradually poured in until the precipitate first formed redissolves, excess of alkali being avoided. It was originally stated that this solution has no action on wool and cotton, whilst it readily dissolves silk, but Richardson observed that when heated with the copper solution for 20 minutes (the time necessary to dissolve silk from plush), purified cotton lost from 1 to 1.5% of its weight, and became friable and tended to disintegrate as dust; whilst woollen frabrics, treated in a similar manner, lost from 9 to 16% of their weight. Hence the reagent is useless for the analysis of fabrics containing wool.

From the solution of cotton or silk in ammoniacal copper oxide solution, the addition of salts will cause a precipitate in the case of cotton, but not in that of silk which is only thrown out by dilute acids.

Persoz observed that silk dissolved in a boiling solution of oxychloride of zinc, and on this reaction von Remont based the following method of analysing mixed fabrics containing silk, wool, and cotton. Four quantities (A, B, C, and D) of two grm. each of the air-dried material are weighed out. One of these (A) is kept, and each of the other three boiled for 15 minutes in 200 c.c. of water containing 3% of HCl. The liquid is decanted, and the boiling repeated with more dilute acid. This treatment removes the size, and in some cases the colouring matter. Cotton may be decolorised very quickly, wool less readily, and silk but imperfectly. Light shades on silk can be neglected, but the black dye on silk often forms a considerable part of the weight of the fabric. Its incomplete removal is indicated by the ash left on igniting the fabric containing iron, a test which is more reliable than the incomplete decolorisation of the stuff, since this may occur when only an insignificant amount of colouring matter remains. The acid is removed from the material by washing and pressing, and portion B is then laid aside. To remove the silk, portions C and D are then next placed for 2 minutes in a boiling solution of basic zinc chloride of 1.72 sp. gr.,[1] then thrown into water, and washed first with water acidified with 1% of hydrochloric acid and then with distilled water until the washings are free from zinc. Portion C is pressed and laid aside. For the separation of the wool and cotton D is boiled gently for 15 minutes with 60 to 80 c.c. of sodium hydroxide solution of 1.02 sp. gr., and then washed very carefully in water, care being taken not to destroy the vegetable fibre. The 4 portions, A, B, C, and D, are then dried for an hour at 100°, and left till the following day fully exposed to the air, so that they may absorb the normal amount of hygroscopic moisture. If a, b, c, and d are the weights of the portions represented by the corresponding capital letters, then:

$a - b$ = dye and finish; $b - c$ = silk; $c - d$ = wool; and d = vegetable fibre. Some analysts make an allowance of 5% on the weight of d, to compensate for loss by the action of the alkali. In the case of a black silk resisting the action of dilute acid, its composition can be arrived at by the method described on page 226.

The foregoing process has been critically examined by Richardson (*J. Soc. Chem. Ind.*, 1893, **12**, 430); working on the air-dry material, and subsequently exposing the treated portions overnight to the air,

[1] The reagent is prepared by dissolving 1,000 grm. of zinc chloride in 850 c.c. of hot water, adding 40 grm. of zinc oxide, and heating the mixture until solution is complete.

is apt to lead to misleading results; therefore the material should be thoroughly dried at 100° before being weighed, and the treated portions weighed after being dried at the same temperature.[1] Boiling with water containing 3% of hydrochloric acid acts too generally on the wool and cotton to render such treatment desirable. Boiling for 10 minutes with 1% hydrochloric acid is more satisfactory. Richardson dipped the material two or three times into the boiling zinc chloride solution, taking care that the total time of immersion did not exceed 1 minute. It is necessary that the zinc solution should be sufficiently basic and concentrated to obtain satisfactory results. Even then, purified cotton loses about 0.5%, and purified wool from 1.5 to 2.0% of its weight.

An ammoniacal solution of nickel oxide has been recommended by Richardson (*J. Soc. Chem. Ind.*, 1893, **12**, 430) as a reagent for separating silk from wool and cotton in mixed fabrics.[2] Silk dissolves rapidly even in the *cold*, and after treatment for 2 minutes, which is the time necessary to dissolve the silk from fabrics other than plush, purified cotton lost 0.45, and purified wool 0.33%. In order to dissolve silk from plush, it is necessary to boil the material with the nickel solution for 10 minutes under a reflux condenser. Cotton loses 0.8% of its weight when thus treated. In Dreaper's hands this method has given satisfaction.

For the analysis of *plush*, Richardson recommends a brief exposure of the fabric to the action of a boiling solution of basic zinc chloride, as already described, but for the determination of silk in light fabrics, especially when wool is also present, exposure from 1 to 3 minutes *(according to the nature of the material) to a cold solution of ammoniacal* nickel oxide is to be preferred.

Richardson mentions the case of a plush which contained rather more silk than cotton, and gave the results shown on p. 816.

Plush backs are much harder than most cotton fabrics, and successive treatment with acid and with the copper and glycerin reagent gives good results; but the copper reagent is not suited for use with other cotton goods, and wool is dissolved by it to a very serious extent. Richardson found a mixed woollen and silken fabric, after

[1] As textile fibres are hygroscopic, the usual precautions must be adopted to prevent the material from reabsorbing water during weighing.

[2] The reagent is best prepared by dissolving 25 grm. of crystallised nickel sulphate in about 80 c.c. of water, 36 c.c. of a 20% solution of sodium hydroxide is then added, and any excess of alkali carefully neutralised by dilute sulphuric acid. The precipitated nickel hydroxide is redissolved in 125 c.c. of strong ammonia, and the solution made up with water to 250 c.c.

	By ammoniacal nickel solution	By basic zinc chloride solution	By copper glycerin reagent
Moisture, dye, and finish....	11.34	11.00	10.04
Silk	45.60	45.00	47.06
Cotton	43.06	44.00	42.90
	100.00	100.00	100.00

being thoroughly cleansed from extraneous matter and dried, gave with the nickel reagent 25.4% of silk, and with basic zinc chloride 27.0%. He considered the former result the more accurate, as the zinc solution acts to a greater extent on the wool.

Fibre	Fibres actually present; %	Percentage obtained by		
		Ammoniacal nickel solution	Basic zinc chloride solution	Copper-glycerin reagent
Silk..........	5.84	5.92	5.52	18.80
Wool.........	76.31	76.58	80.08	64.05
Cotton.......	17.85	17.50	14.40	17.15
	100.00	100.00	100.00	100.00

In fabrics containing silk, wool, and cotton, the silk may first be dissolved by treatment with the nickel reagent. The portion left insoluble is first treated with very dilute hydrochloric acid (1%), and the wool then dissolved by boiling for 7 minutes with a 2% solution of sodium hydroxide. The foregoing results were obtained from a material which had been very imperfectly dyed, and the constituent fibres of which were easily separated and weighed. It will be observed that fairly accurate results were obtained by the nickel reagent, but not by the copper nor the zinc solution.[1]

The ammoniacal nickel solution has been employed by Merritt Matthews with satisfactory results. The following are the details of procedure, which differ in certain respects from those given above:—The yarn or fabric is cut up very fine with a pair of scissors, and thoroughly dried at 100°. 1 grm. of the material thus prepared is treated with 40 c.c. of the cold ammoniacal nickel solution for 2 min-

[1] Richardson considered the solvent action of basic metallic solutions on silk to be due to dissociation of the amino-anhydride of which the fibre is composed, with formation of soluble metallic amino-compounds.

utes. The liquid is then filtered, and the residue, consisting of the wool and cotton of the sample, is digested for 2 or 3 minutes in 1% boiling hydrochloric acid, washed free from acid, dried at 100°, and weighed. To dissolve the wool from the cotton, the residue is boiled with about 50 c.c. of a 1% solution of potassium hydroxide for 10 minutes, and the solution filtered. The residue, which consists of *cotton*, is washed free from alkali, dried at 100°, and weighed.

The foregoing method assumes that silk-gum, dyes, and weighting materials have been previously removed. To remove the silk-gum, and possibly the dye, the material may be boiled in a 1% solution of soap for one hour, but Richardson has considered that its complete removal is best effected by treatment in the cold with a 2% solution of potassium hydroxide. This has the advantage of decomposing Prussian blue, and facilitating the subsequent removal of iron by 1% hydrochloric acid. After this last treatment the material should be thoroughly washed and dried. Metallic mordants are apt to be imperfectly removed, and their amount must be deduced from the ash left on ignition and subtracted from that of the other constituents. It is sometimes advisable to boil dyed fabrics with methylated spirit and then with ether, to remove certain dyes and oily matters.

No general method for the removal of colouring and weighting materials from fabrics can be suggested. General information on the subject will be found in the following pages.

Recognition of Tussah Silk.—The published tests which distinguish ordinary silk (*Bombyx mori*) from tussah cannot be considered as altogether satisfactory, but Dreaper placed reliance on the results obtained in the following general examination:

Microscopical appearance of the fibre as compared with ordinary silk.

Millon's reaction, which gives a chocolate brown colour with degummed Tussah, instead of red as with ordinary silk.

Amino Acid Test, which gives a deep brown with degummed Tussah, in place of bright red with ordinary silk.

Solubility in Nitric Acid (conc).—Tussah silk, as present in a fabric (degummed), dissolves on boiling for 30 seconds and gives a darker colour to the solution than ordinary silk.

Solubility in Hydrochloric Acid (conc) or 5% Sodium Hydroxide Solution.—Ordinary silk completely dissolves in each case, on boiling,

within 5 minutes. Tussah silk does not dissolve, but the filaments become very transparent as compared with cotton, which latter fibre may actually powder down in the acid solution (see page 215).

Dyeing and Weighting Silk

Silk has a great affinity for the coal-tar colours, which dye it without the use of any mordant, though it is customary to use a soap-bath (boiled-off liquor), with or without the addition of acetic or other weak acid. Indigo-sulphonic acid is used for indigo tints. For black, the silk is treated with ferric acetate, washed and immersed in a bath of potassium ferrocyanide, followed by another iron bath, washing, and immersion in a catechu or other tannin bath, followed by one of logwood and soap.

Methods for the qualitative investigation of dyestuffs on animal fibres are given by Fierz-David and Yarsley (Vol. VI, 619 *et seq.*).

Determination of Weighting in Silk

In addition to legitimate processes of dyeing, silk is subjected to operations having for their object the introduction of weighting materials. This practice of adulteration is carried out systematically and to an extraordinary extent, the adulterants sometimes amounting to more than the weight of the true silk.[1]

Carter Bell (*J. Soc. Chem. Ind.*, 1897, **16**, 304) gives the following analyses of unweighted and weighted silk.[2] The standard taken for silk was pure boiled-off silk, dried at the ordinary temperature, and assumed to contain 18% of nitrogen.

[1] The history, objects, and effects of silk-weighting have been fully discussed by Wardle and Carter Bell (*J. Soc. Chem. Ind.*, 1897, **16**, 297).

M. J. Langdon (*J. Soc. Chem. Ind.*, 1897, **16**, 405) suggested that the adulteration of silk by weighting should be limited by arrangement between the dyers to the comparatively moderate amounts of 16 to 84 ounces for 16 ounces of silk, according to the nature of the material.

J. Carter Bell (*J. Soc. Chem. Ind.*, 1897, **16**, 303) has described a case in which 100 pounds of silk were sent to the dyer with the request that he would weight it to 1,000 pounds. This being done, the weighted silk contained 9% of moisture, under 2% of nitrogen (corresponding to less than 10% of silk), and yielded 43% of ash.

In 1897, the silk-weavers of Zurich entered into an agreement for one year with the Swiss silk-dyers, forbidding, on pain of heavy fines, the weavers of import or use, and the dyers to dye, any silk, whether of home or foreign origin, that had been weighted, to such an extent as seriously to impair the strength of the fibre. Following this example, the silk-dyers of Crefeld (Germany) agreed that the maximum degree of weighting in the future should be 30 to 40% for taffetas, and 50 to 60% for other kinds of silk (*J. Soc. Chem. Ind.*, 1897, **16**, 532).

Highly-weighted silk burns without flame when heated. It smoulders slowly away, leaving the original form intact. Instances of the spontaneous combustion (smouldering) of black silk are on record (*J. Soc. Chem. Ind.*, 1897, **16**, 304).

[2] The practice of weighting silk probably had its origin in the desire to make up the loss of weight (amounting to about 25%) which occurs in the boiling-off process, and competition has increased the amount of weighting materials introduced till they have reached the present proportions.

	Moisture	Ash	Silk
1. White silk	5.1	0.340	100
2. Salmon-coloured Shanghai	5.5	0.246	100
3. Black	7.9	18.7	25
4. White	8.2	50.0	25
5. Shot silk	6.2	34.7	39
6. —	10.2	33.7	32
7. Pink silk	8.5	43.2	54
8. Pink silk	9.4	52.4	43
9. Pink silk	8.1	49.5	46
10. Blue	6.5	35.4	60
11. Gold colour	5.8	41.7	52
12. Cream colour	9.1	45.7	46
13. Pink and green	8.6	34.2	50
14. Pink	8.6	28.2	60
15. Cream	0.3	8.9	87
16. Yellow	8.6	9.1	86
17. Black	8.5	42.9	12
18. Sky-blue	0.1	48.0	37
19. —	8.1	45.9	36

The sp. gr. of silk has been used by Vignon (*Bull. Soc. Chem.*, 1892, **7**, 247) as a means of ascertaining the proportion of weighting materials present, but the practical utility of the method is lessened by the necessity of knowing the nature of such weighting materials. Vignon ascertains the sp. gr. of the fibre, in its ordinary air-dry condition, in benzene, after removing occluded air by means of the air-pump. The sp. gr. of some textile fibres were thus determined by Vignon and by Silbermann (*Chem. Ztg.*, 1904, **18**, 744) with the following results: Wool, 1.28 to 1.33; cotton, 1.50 to 1.55; mohair (combed), 1.30; hemp (carded), 1.48; ramie, 1.51 to 1.52; linen (spun), 1.50; jute (spun), 1.48; silk (raw), 1.30 to 1.37; boiled-out silk, 1.25. The sp. gr. of silk varies in course of treatment by dyeing, bleaching, and weighting, and in course of mechanical treatment by stretching, soupling, etc.

In order that data as to the approximate amount of weighting present in any given silk may be deduced from its sp. gr., the nature of such weighting and the process of dyeing used must be known, at least approximately.

The theory of silk weighting has been fully discussed by Heermann (*Chem. Ztg.*, 1911, **34**, 829). See also Fichter and Muller (*Arch. Sci. phys. Nat.*, 1916, **42**, 123) Fichter and Reichart, (*Helv. Chim. Acta*, 1924, **7**, 1078) who consider that in the case of weighting with

stannic chloride a true chemical compound is formed with the fibroin.

The materials used for weighting silk are of varied character. The silk may be immersed in some solution of a metallic salt and then transferred to a bath of salt which will react to form an insoluble precipitate on or in the silk-fibre. Thus iron is precipitated as ferrocyanide, gallotannate, and catechu-tannate; tin as catechu-tannate, tungstate, phosphate, silicate, oxide, etc. The use of tin in the form of diammonium stannichloride, $(NH_4)_2SnCl_6$, commonly called "pink salt," and its precipitation in the fibre as phosphate and silicate, has increased enormously of late years. Chromium compounds are also used, besides sulphates of sodium, magnesium, and barium, and various organic matters (*e. g.*, gelatin, dextrose, logwood, and tannins).[1]

Silk may be weighted with tin in the following manner, and this may give the analyst some idea as to the nature of the weighting material present on the yarn. The silk is soaked for 1 hour in stannic chloride solution of 30–35° Bé. After washing, it is introduced into 4° Bé bath of sodium phosphate in which it is heated to boiling. It is then heated in a 5° Bé. aluminium sulphate bath for 15 minutes, and this is followed by a warm bath of 5° Bé. sodium silicate. This sequence of operations is repeated as often as desired, great care being taken to wash the yarn thoroughly in water between each operation. The final bath is one of a very fat soap at 30°. In the case of tannin weighting in light colours the silk is steeped in a 4° Bé. pure gall extract (bleached).

In some cases it is necessary to identify and separately determine the various weighting materials present in a sample of silk, but in other cases it is sufficient to know their total weight. For the estimation of such additions as oil, sugar, soap, etc., 5 grm. of the sample of silk should be dried at 100°, and then extracted in succession with ether, alcohol, and boiling water; the material is dried and the loss of weight noted after each treatment.[2]

[1] Salts of nearly all the commoner metals have been proposed for use as weighting materials. Detailed information on the methods of weighting silk will be found in articles by Sibermann (*Farb. Zeit.*, 1896, **8**, 34, 51, 68).

[2] Silbermann (*Chem. Ztg.*, 1894, **27**, 744) recommends the following method for the recognition of the nature of the weighting and dyeing materials present in silk: Readily-soluble materials, such as cane-sugar, dextrose, glycerin, magnesium salts, etc., are estimated directly by boiling the silk with water, and testing the extract with Fehling's solution, etc. From 2 to 3 grm. of silk are ignited, and the ash tested for tin (present in the fibre as basic chloride and stannic acid), chromium, iron, etc. Fatty matters, wax, and paraffin wax are detected by extraction with ether or benzene. The silk is soaked in warm, dilute hydrochloric acid (1:2). If the fibre is almost decolorised by this treatment, only a slight yellow tint remaining, while the solution assumes a deep brownish colour *not* changed to violet by lime-water, it is safe to conclude that the silk has been dyed by alternate passage

The percentage of weighting materials is usually expressed on the raw silk, and not on the boiled-off silk. As the raw silk loses 25% of silk-gum in the process of degumming, it follows that 75 parts of conditioned silk represent 100 of raw, and this amount is allowed for in stating the proportion of weighting materials found.

The increase of the volume of silk by dyeing is in practice more important than the increase of weight, and thus the desired effect is produced better by the use of tannin than by the introduction of excessive proportions of metallic compounds.

In examining *white silk*, the total *soluble weighting materials* should be determined by treating a known weight of the sample 4 or 5 times with hot water, washing it thoroughly, and drying it. In consequence of the great and variable hygroscopic character of silk, to obtain reliable results it is desirable to determine this. After treatment with water and re-drying, the samples are placed together under a clockglass, and when the standard silk has regained its original weight and washed sample is reweighed, the loss represents the matters soluble in water. An aliquot part of the solution may be evaporated to dryness and the residue weighed, to obtain a direct estimation of the matters dissolved. Unweighted silk loses, on the average, 25% of its weight on boiling off, so that only the loss in excess of this must be regarded as due to weighting, unless the additions be actually identified and determined by other means. *Dextrose* may be directly determined in the solution by Fehling's solution, and *cane-sugar* after inversion. *Sulphates* and *chlorides*, *magnesium*, etc., may be detected and determined as usual. *Stannic oxide*

through baths of iron and tannin. The yellow colour of the fibre is due to a residuum of tannin, and the precise shade (from greenish to brownish-yellow) enables a rough idea to be formed as to the nature of the tanning material used (sumach, divi-divi, catechu, etc.). Decolorisation of the fibre, the acid extract being pink, changed to violet by lime-water, indicates a logwood-black (so-called "English black"). If the fibre retains a deep greenish tint and the solution is yellow and unaffected by lime-water, the black is dyed on a Prussian blue ground. If the latter, as is often the case, has been produced during the final stage of dyeing, this will be shown by its solubility in the acid. A green fibre and pink solution, altered to violet by lime-water, point to a logwood-black on a Berlin-blue ground. In the hydrochloric acid solution, metals, such as lead, tin, iron, chromium, and aluminium, may be determined. Blacks produced by artificial dyes on an iron-tannin or iron-blue catechu ground, are recognised by the coloration imparted to acid and caustic soda solutions. As regards blacks produced solely by the agency of aniline-dyes, etc., treatment with a hydrochloric acid solution of stannous chloride does not affect aniline and alizarine blacks; naphthol-black is altered to a reddish-brown colour, while wool-black becomes yellowish-brown. Aniline and alizarine blacks may be distinguished by means of sulphurous acid, which attacks only the former, turning it greenish. Tannin substances, in general, may be extracted by alkaline solutions and subsequently precipitated and distinguished by ferric acetate. To remove the whole of the weighting material and dye, it is recommended to boil the silk with acid potassium oxalate, wash with dilute hydrochloric acid, and finally treat with sodium carbonate solution. When iron and tin are both present in the fibre, it is well to extract the tin previously by means of an alkali-metal sulphide. In conclusion, Silbermann gives a number of experimental results, which exhibit the relation existing between the density of silk and the percentage and nature of the loading material. See also Vignon, *Bull. Soc. Chim.*, 1892, 7, 249).

will be left on igniting the silk in porcelain after the above treatment. In its presence, the material burns with difficulty, and the residue retains the shape of the original silk. The weight of the ash (assuming it to be wholly SnO_2) may be calculated to the form in which the tin exists in the weighted silk, namely SnO_2,H_2O, by multiplying it by the factor 1.12.[1] It may be identified as stannic oxide. The possibility of the tin being present as insoluble compounds must also be considered.

The general estimation of weighting in silks is dealt with by Persoz, (*Rev. Gen. Mat. Col.*, 1906, **9**, 322).

For the further examination of white silk, Silbermann (*Chem. Zeit.*, 1896, **20**, 472) recommends the following procedure:

A weighed portion of the silk is boiled with dilute hydrochloric acid to decompose any tannin-lakes of tin or other metals, and in the solution tannin is tested for by the addition of an excess of sodium acetate and a ferric salt. If tannin-lakes are present the determination of the weighting materials consists in: (1) Removing tannin from the aqueous solution with gelatin; (2) estimation of tannin in this precipitate, and of sugar, etc., in the filtrate; (3) successive treatment of the silk with dilute hydrochloric acid and sodium carbonate, and precipitation of tannin from both solutions by means of "gum" solution (gelatin?); (4) ignition of the silk and determination of metallic weighting. If the ash is not completely soluble in hot, moderately concentrated hydrochloric acid, it may contain barium sulphate or silica.[2]

To calculate the percentage of weighting material, W, in the silk examined, the following formula is employed, in which "a" is the weight of the sample before treatment, "b" the weight after extraction with water, "p" the SnO_2 left on ignition, and "d" the loss of weight during the boiling of the fibre itself. This is taken at 20 to 25 for boiled-out silk ("cuit"), 5 to 9 for souple silk, and 0 to 2 for écru:

$$W = \frac{a(100 - d)}{b - 1.13p} - 110$$

Dark-coloured and black silk may contain hydroxides of tin, iron, and chromium, fatty matters, tannin, Prussian blue, and various

[1] A simpler method for the detection of tin is to heat the silk with just sufficient hydrochloric acid to effect its complete solution, dilute the liquid with water, filter, and pass hydrogen sulphide, when any tin will be precipitated as yellow stannic sulphide.

[2] The ash may also contain stannic phosphate and tungstic oxide. It is doubtful whether treatment with hydrochloric acid can be relied on to remove stannic oxide, and actual fusion in the ordinary manner with suitable salts may be necessary.

other colouring matters. Fatty matters may be removed and determined by treatment with ether. Treatment of the silk with hydrochloric acid (1.07 sp. gr.) at 50 to 60° dissolves the logwood and leaves the silk of a maroon colour in the absence of Prussian blue, or blue black if it is present. In the latter case, on then treating the silk with dilute caustic soda, a solution containing ferrocyanide will be obtained, which will yield a precipitate of Prussian blue when acidified with hydrochloric acid and treated with ferric chloride. The metallic oxides will be contained in the ash left on igniting the silk, and are best examined by fusing the residue in platinum or silver with nitre and sodium carbonate and treating the product with water, when the tin and chromium will be dissolved as stannate and chromate, respectively, and the iron will remain insoluble. From the acid filtrate the tin may be precipitated by hydrogen sulphide, and the chromium thrown down from the filtrate by ammonia, or determined by other known means. For the detection of free *tannin*, a portion of the sample should be boiled with water, and a small quantity of ferric acetate solution added, when a blue-black liquid is produced in presence of tannin. Or the sample may be boiled with very dilute hydrochloric acid, and the liquid tested with gelatin solution or ferrous sulphate. Free tannin may be determined by dissolving it from the silk by passing it through an alkaline soap bath at 95°, and finding the loss of weight on redrying.[1]

For a rapid determination of the *total proportion of weighting materials*, a known quantity of the silk, previously dried at 110°, should be boiled for 10 minutes with a 2% solution of sodium carbonate and then with dilute hydrochloric acid (250 grm. of the commercial acid per litre). This treatment is repeated up to four times, washing the material between each treatment. Experience will indicate the treatment that silks will stand in this direction. The silk, which will now have become very brittle and must be carefully handled, is dried at 110°, and weighed.

If souple or écru silk be under examination it should be subjected to a final boiling with soap before drying.

[1] The following is an outline of the method of analysing weighted silk recommended by E. Königs, Director of the Silk-Conditioning Establishment at Crefeld: (1) Estimate moisture by drying. (2) Fatty matters by extraction with ether. (3) Boil out silk-gum with water. (4) Dissolve out Prussian blue with alkali, reprecipitate with acid and ferric chloride, and ignite precipitate with addition of HNO_3; part of Fe_2O_3 = 1.5 parts of Prussian blue. (5) Estimate SnO_2 present in ash of silk and calculate as catechu-tannate of tin; one part of SnO_2 = 3.33 parts of catechu-tannate. (6) Estimate total Fe_2O_3, subtract that present as Prussian blue, and the amount naturally in the silk (0.4 to 0.7%), and calculate the remainder to tannate; 1 part of Fe_2O_3 = 7.2 parts of ferric tannate (or 5.1%, if present as a ferrous compound).

By the foregoing treatment all foreign substances are removed, except mere traces of tannin and colouring matters, and in some cases small quantities of metallic compounds. The weight of the ash left on igniting the treated silk, if multiplied by 1.25, will represent pretty nearly the hydrated metallic oxides retained in the washed silk.

A certain loss of silk-substance occurs in the treatment, and hence the proportion of weighting materials found in the sample is somewhat in excess of the truth. But the chief source of error lies in the uncertainty of the allowance to be made for loss in the weight of the silk by "boiling off." For boiled-off silk this (d) is taken at 25%; for souple silk at 8%; for écru at 0. If p be the original weight, and D the weight after boiling, the percentage of weighting, W, may be found by the following equation:

$$W = \frac{(100 - d) \times (p - D)}{D}$$

In cases where the treated silk leaves a sensible quantity of ash, a, on ignition the following equation must be substituted:

$$W = \frac{(p - D + 1.25a) \times (100 - d)}{D - 1.25a}$$

The foregoing process is tedious and not very accurate. A preferable plan is to determine the total nitrogen by Kjeldahl's process, after removing any gelatin, Prussian blue, or other extraneous substances containing nitrogen. This is effected by boiling a weighed quantity of the silk (1 or, preferably, 2 grm.) with a 0.2% solution of sodium hydroxide for ½ hour.[1] The silk is then removed, washed, and heated to 60° for ½ hour in water containing 1% hydrochloric acid, and well washed with hot water. The treatment should be repeated until the silk no longer retains a blue colour. In the case of souple and écru silks, ammonia or ammonium carbonate should be substituted for sodium hydroxide and the discharged silk should be subjected to a final boiling for an hour with a 1% soap solution to remove any silk gum present.

Air-dried silk with 11% of water contains 17.6% of nitrogen, and hence the silk in a sample free from Prussian blue, etc., can be found by multiplying the percentage of nitrogen by 5.68.

[1] Prussian blue may be removed by boiling the silk in a solution of acid oxalate of potassium.

Utz (*Chem. Ztg.*, 1923, **47**, 36) uses, however, a slightly lower protein factor in calculating the amount of fibroin in silk. His method of analysis is to dry the sample at 105 to 120°, to determine the moisture content. The bast content is measured by the loss in weight brought about by digesting the dried material for 70 minutes in a 0.75% solution of neutral olive oil soap, and washing the material three times with distilled water before drying. The fibroin is determined by boiling 1 to 2 grm. of the sample for 2 hours in a 2.5 to 3.0% solution of soap and then further boiling with a solution of sodium carbonate (Sp. gr. 1.01) until all volatile ammonia is removed. The nitrogen in the residue is determined by Kjeldahl's method, 1 mg. of nitrogen being equivalent to 5.455 mg. of fibroin. The amount of loading is determined by difference.

Estimation of Prussian Blue.—The actual estimation of Prussian blue may be carried out by the following means (Williams and Dreaper, *J. Soc. Dyers and Col.*, 1912, **28**, 336), whereby the whole of the nitrogen in the Prussian Blue is converted into hydrocyanic acid and distilled off into *N*/10 sodium hydroxide solution.

The actual process is as follows: A weighed quantity of the silk is distilled with a convenient quantity of 10% solution of sulphuric acid to which is added 0.1 grm. of pure cuprous chloride dissolved in a few drops of concentrated hydrochloric acid. The hydrocyanic acid is distilled into *N*/10 sodium hydroxide (or even *N*/100) and the solution afterwards treated with *N*/10 silver nitrate. The number of c.c. of *N*/10 silver nitrate required, plus 0.01355, represents the actual weight of hydrated Prussian blue present.

The accurate estimation of the Prussian blue has the further advantage of yielding under certain conditions a correspondingly exact figure for the pure silk fibre present. By deducting the calculated amount of nitrogen present in the Prussian blue, from the total nitrogen, as usually determined by Kjeldahl's method, this figure represents in the known absence of any nitrogenous dressing or "silk gum" the nitrogen due to the silk (fibroin) itself.

Any insoluble, or other dressing material, and "silk gum" present must be removed by boiling for, say, half an hour in 2% sodium carbonate solution (or preferably, 1% sodium carbonate solution and 1% soap solution) which will also remove the Prussian blue.

In the case of a sample of raw silk dyed black, this last treatment must be repeated. In this way nitrogen figures for the silk, the

Prussian blue, and also, possibly, any nitrogenous dressing or "silk gum" present, can be obtained.

The following results have been obtained in practice:

EXPERIMENTAL RESULTS

(1) Black weighted silk distilled direct with dilute sulphuric acid and cuprous chloride =

$$\begin{matrix} Fe_4^{III}(Fe(CN)_6)_3 & & Fe_4^{III}(Fe(CN).2H_2O) \\ 3.68\% & = & 5.22\% \end{matrix}$$

(2) The same extracted repeatedly with a 2% solution of sodium carbonate, a portion of the extract distilled with dilute sulphuric acid and cuprous chloride =

$$\begin{matrix} Fe_4^{III}(Fe(CN)_6)_3 & & Fe_4^{III}(Fe(CN).2H_2O) \\ 3.67\% & = & 5.21\% \end{matrix}$$

(3) The above sodium carbonate extract acidified and precipitated with an iron salt in excess, filtered, washed, and ignited = 2.77%-Fe_2O_3 =

$$\begin{matrix} Fe_4^{III}(Fe(CN)_6)_3 & & Fe_4^{III}(Fe(CN).2H_2O) \\ 4.25\% & = & 6.03\% \end{matrix}$$

$$Fe_4^{III}(Fe(CN).2H_2O)$$

(4) Above (3) repeated = 4.25%

In the known absence of "silk gum" or soluble or insoluble dressing material, the Prussian blue may be estimated by the difference in the percentage of nitrogen present, before and after extraction of the Prussian blue from the fibre by a suitable alkali treatment (2% sodium carbonate solution) at boiling point, which may be repeated if necessary. This process is recommended by (Farrell and Goldsmith, *J. Soc. Dyers and Col.*, 1912, **28**, 236). It must be remembered, however, in calculating results that anhydrous Prussian blue is unknown and it is best to average the water of hydration at 20 molecules under the usual conditions of silk dyeing.

The determination of nitrogen must be conducted with great care.[1]

[1] *In the analysis of weighted silks Müller recommends removing the weighting material* with a 0.4 to 1% solution of hydrofluoric acid or a saturated solution of oxalic acid (*Z. Farb. u. Text. Ind.*, 1903, 160).

Gnehm *and Blumer treat the* silk first with hydrochloric acid at 60° C., and subsequently with sodium carbonate at 80°, repeating both the treatments several (up to seven) times. (See also Gnehm and Schwartz, *Rev. Gén. Mat. Col.*, 1898, **2**, 131.)

Silk fabrics should be separated into warp and weft, and these analysed separately, since the weft is usually more heavily weighted than the warp.

It is a frequent practice to express the proportion of weighting materials on 100 parts of the silk treated, and not on 100 parts of the product. This plan is adopted in the following statement of results obtained by Gnehm and Blumer (*Rev. Gen. Mat. Col.*, 1898, **2**, 13?) by a modification of the foregoing process.

	Weighting above pari	
	Actual	Found
A. Japanese trame,[1]	96%	95.71%
B. Japanese trame,[1]	50%	49.66%
C. Yellow Italian organzine,[1]	53%	52.81%

[1] *Traume* or *tram*, usually employed for wefts, is the product of the union of two or more single untwisted threads, which are then doubled and slightly twisted. *Organsine*, generally used for warp-silk, is produced by the union of two or more single threads separately twisted in the same direction, and then doubled and re-twisted in the opposite direction.

Artificial Silk or Rayon (Glanzstoff)

Natural silk is probably the most highly prized of all textile materials, but on account of its high price the problem of simulating it has attracted attention for a considerable time. Hooke (1667) probably made the first suggestion as to a means of producing fine artificial fibres, while Réaumur (1734) suggested that solutions of resins and gums could be drawn out in fibres of requisite fineness. It was not until 1855 that a process was elaborated and patented for the use of cellulose nitrate dissolved in a mixture of alcohol and ether for the preparation of artificial silk (Andemars, *Eng. Pat.* 1855, 283). In 1882, the discovery by Schweizer that cellulose is dissolved by an ammoniacal solution of copper hydroxide was applied by Weston and, later commercially by Swan (1883), for the purpose of producing fine filaments. Chardonnet (1891), who used the cellulose nitrate method, is generally considered the real founder of the artificial silk industry, although Swan had used this method previously. In 1897, Fremery and Urban successfully applied the cuprammonium method, but the importance of this type of artificial silk has been overshadowed by the viscose rayons. At the present time, the viscose process is undoubtedly the most important, about

85% of the production of artificial silk being made by this method. In this process, which was discovered by Cross, Bevan and Beadle in 1892, the cellulose is treated with caustic soda and carbon disulphide and forms a soluble orange-yellow viscous substance which is the sodium salt of the cellulose ester of dithiocarbonic acid. The ester is often termed cellulose xanthate, and the silk spun from it "xanthate silk."

The importance of the artificial silk industry is well indicated by the remarkable growth in output which has taken place during the fourteen years 1913 to 1927, when the annual output rose from 11,550 to 120,600 metric tons. Artificial silk cannot, of course, be considered as a true structural protein, as it is not a natural product and the material from which it is made is not a protein. Owing to its colloidal nature, however, it has many of the properties of the true structural proteins.

The artificial silks made by the above processes, when wet, only possess about 50% of the dry strength, and, in order to overcome this, considerable attention has been directed to the production of "acetate silk" from cellulose acetate. This type of artificial silk is, however, rather expensive, and it has not been able to supplant the viscose variety.

Founded on the work of Strehlenert, many attempts have been made to increase the resistance of other types of artificial silk to water by treating them with formaldehyde. Several patents have been taken out on these lines, and all claim that the tensile strength of the artificial silk is greatly increased by such a treatment. This treatment has been termed "sthenosage." Unfortunately, however, the process affects the rayons in other directions, decreasing their elasticity and increasing the difficulties in obtaining level shades in dyeing. To some extent, this difficulty of dyeing can be overcome, but, owing to the expense of the process, very little, if any, artificial silk is, at the present time, treated with formaldehyde. In the case of viscose, the modern method employed for increasing its "wet strength" is to stretch spin it into a bath of fairly concentrated sulphuric acid—up to 60% concentration. Artificial silk so treated is termed "Lilienfeld" rayon.

The newest form of artificial silk is made from cellulose ethers, but it is not yet produced in very large quantities. Efforts have been made to produce artificial silks from materials other than

cellulose. The use of plant mucilages, agar agar, etc., casein, gelatin, keratin and rubber (Marriott, Eng. Pat. 205532) have been suggested, but, apart from a small amount of artificial silk produced from gelatin, these methods have not been successful. Attempts have been made to produce a "glass silk" by drawing out glass, but, owing to its brittleness, it is unsuitable for textiles. Occasionally, however, it has been used for ornamenting ties.

The general procedure in the manufacture of artificial silk or rayon, as it is now generally called, is to treat the cellulose in such a way that it can be dissolved to form a viscous solution, and this, after filtering, is expressed through fine orifices in much the same way that a spider expresses fibroin through its spinnerets. The fine fibres which exude are then hardened, either by drying or treatment with different hardening solutions, and then suitably spun into yarns. In some cases, the exuded fibres are subjected to tension before they have hardened; this process is called "stretch spinning," producing a thinner fibre. Where nitro-cellulose has been used, a denitration bath, usually consisting of a solution of crude calcium hydrosulphide, is employed to reduce the inflammability.

The raw materials available for the production of rayon vary according to the process which is to be used. Thus, cotton is the only suitable material for the nitro-cellulose process and is to be preferred above other substances in the cuprammonium process, although during the War, sulphite cellulose was used. It is here, however, that the viscose process gains an economic advantage, since the raw material is almost entirely wood cellulose. All types of sulphite cellulose are not equally suitable; the limits of the content of α- and β-cellulose should lie between 86 and 89% and 6 to 8%, respectively. In the making of nitro and acetate artificial silks, cotton linters are used as the raw material.

Ordinary artificial silk consists of practically endless fibres, but efforts have been made, especially during the War, to make use of shorter lengths by attempting to imitate Chappe silk, which is made up of short lengths of natural silk which are spun into a yarn. One of the disadvantages of ordinary artificial silk is that it does not retain the heat of the body, but this is remedied to a great extent by spinning short lengths (about 10 cm.) of rayon. Such material is known as "staple yarn." By spinning staple fibre (*i. e.*, short lengths of artificial silk) in admixture with wool and cotton, a

pleasing material is produced, which is used for weaving and for knitting.

Properties of Artificial Silks.—Bleached artificial silk is more or less pure white, the individual fibres being clear. Viscose rayons, which have not been desulphurised, are generally dark cream coloured. Cellulose nitrate rayons are usually more lustrous than the viscose variety, while cuprammonium rayons are least lustrous and exhibit small glittering points except in those types which consist of very fine filaments. Some rayons are specially made, however, with a matt surface. The amount or strength of the lustre can be determined with a "Lustremeter."

Like all organic fibrous material, artificial silk can absorb considerable quantities of water without feeling damp. From the commercial point of view, the water content is important, and the accepted method of determining this is given later.

Recognition of the Type of Artificial Silk.—Artificial silk can be recognised by its solubility in organic solvents (acetate rayon) or by burning or by measuring the tensile strength before and after wetting, but in differentiating between the various types the microscope is of great assistance.

Chemical Properties

Copper Numbers.—When cellulose is heated in Fehling's solution, reduction of the copper occurs and the amount of copper in milligrams reduced by 100 grm. of the sample is called the "copper number." This test, due to Schwalbe, suffers from a number of defects and has been superseded by that of Braidy. In Braidy's method, the Fehling solution is replaced by a solution of copper sulphate made alkaline with a sodium carbonate and bicarbonate mixture. The solutions used are:

(a)	Copper sulphate, $CuSO_4.5H_2O$	100 grm.
	Water	to 1 litre.
(b)	Sodium bicarbonate	50 grm.
	Sodium carbonate (crystalline)	350 grm.
	Water	to 1 litre.

Five c.c. of solution "a" are added to 95 c.c. of solution "b" and the mixture heated to boiling and poured over 2.5 grm. of the rayon, which should be contained in a conical flask of a capacity only slightly in excess of 100 c.c. After gentle stirring to get rid of air bubbles, the flask is immersed in a bath of boiling water, the mouth being closed by means of a suitable loose stopper. After three

hours' treatment, the contents are rapidly filtered, and the rayon, which is impregnated with cuprous oxide, is washed with dilute sodium carbonate solution and then with hot water.

The cuprous oxide is then dissolved by treatment with an acidulated solution of iron alum (iron alum 100 grm., concentrated sulphuric acid 140 c.c. made up to a litre with water), three lots of 15, 10 and 10 c.c. usually being sufficient. The cotton is finally washed with 2 *N* sulphuric acid and the combined washings are then titrated with standard permanganate The end-point of the titration is sharp.

The work of Ridge, Parsons and Corner (*Shirley Inst. Mem.*, 1930, **9**, 135) indicates that the copper number for viscose rayon (including staple fibre materials) lies between 0.8–1.2, except in the case of Oburg and Celta (hollow) rayons, the values of which are high (circa 1.6). Lilienfeld rayons (unbleached) give values similar to viscoses. Cuprammonium yarns appear, however, to fall into two classes, (a) 1.0 to 1.5 and (b) 0.5 to 0.6. It is possible that the cuprammonium rayons with the high copper numbers were made from wood pulp, thus giving values of the same order as viscose rayons and rendering any differentiation between cuprammonium and viscose rayons impossible by merely considering the reducing power. Acetate and nitrocellulose rayons have high copper numbers, of the order of 2.8 to 3.2.

A high copper number may be due to non-cellulosic impurities associated with substantially undamaged cellulose in the yarn, or it may result from tendering of the cellulose. The copper number can, however, be diminished by treatment with alkaline solutions which remove degraded cellulose, so that a low copper number may refer to a yarn which has a low tensile strength, due to hydrolysis of the cellulose.

A comprehensive series of data on the copper numbers as well as the moisture regain, ash and sulphur content of various rayons are given by Wahl and Rolland (*Rev. Gén. Mat. Col.*, 1929, **33**, 1).

Fluidity.—The fluidity of a solution of cellulose in ammoniacal copper hydroxide has been used as a means of determining how far the cotton has been attacked by chemical reagents. Ridge, Parsons and Corner (*loc. cit.*) have applied this method to artificial silk. These authors employed similar conditions to those of Clibbens and Geake (*J. Text. Inst.*, 1928, **19**, T77), except that the concentration

of material in the cuprammonium was 2%, as against only 0.5% in the case of cotton. This higher concentration is recommended, since the same apparatus can then be used for both rayon and cotton.

In making the determination, the rayon is best weighed out in a glass tube provided with cords at either end. The contents are pushed out by means of a plunger, made from a piece of glass rod and a rubber band, directly into the viscometer containing the cuprammonium solution. The viscosity of the solution is then determined, taking the usual precautions.

According to Ridge, Parsons and Corner, the fluidity of the solution is, without doubt, the most reliable method of estimating the extent to which the cellulose has suffered chemical attack. These authors give a list of fluidity values for a large series of rayons.

Methylene Blue Absorption.—The amount of methylene blue absorbed by the different rayons is more or less characteristic. Thus acetate rayons absorb less methylene blue than do other varieties; cuprammonium rayons have higher values and are similar to viscose types. Lilienfeld yarns yield high values.

The *p*H value of the solution of methylene blue affects the amount of absorption, and it is advisable to stabilise the *p*H value at 7 by means of a phosphate buffer.

The method is carried out by shaking 2.5 grm. of the rayon with 15 c.c. of the methylene blue solution (about 2.5 to 3.5 millimols per litre) for 18 hours, centrifuging, and estimating the excess dye by means of a solution of naphthol yellow S. The naphthol yellow produces a reddish brown precipitate, and the solution at the end point becomes yellow. Towards the end of the titration the liquor should be centrifuged between each addition. The absorption is calculated as millimoles of dye per 100 grm. of dry material.

The action on rayon of hydrochloric and sulphuric acids of relatively low concentrations at ordinary temperatures causes a decrease in the methylene blue absorption, but when sulphuric acid is used in concentrations of 50% and over, an increased absorption results. This effect of sulphuric acid may be due to the retention of the acid, and probably accounts for the high values given by Lilienfeld yarns. In the case of staple fibres, such as Fibro and Vistra, the high absorptions may be due to the high ash and ash alkalinity of these materials. If the high absorption is due to alkalinity, washing the rayon with

dilute acid brings about a decrease in the value. On the other hand, high absorption values due to combined acid are not reduced to normal or low values, even by boiling with dilute alkali.

By comparing the amount of blue absorbed from solutions at *p*H 7 and 2.7 (made by dissolving the dye in *N*/10 acetic acid) information can be obtained as to whether the high absorption in *p*H 7 has been caused by oxidation (over-bleaching) or by acid attack under conditions where acid is retained by the cellulose. Thus oxycelluloses show a decrease in the amount of dyestuff absorbed from the acid solution, whereas hydrocelluloses have about the same absorptive powers from both acid and neutral solutions of methylene blue.

Ash Content and Ash Alkalinity.—Although the amount of ash is largely dependent on the type of water used in washing the silk, yet the estimation of the ash does serve as a rough sort of guide in identification. According to Kami (*J. Cellulose Inst., Tokyo*, 1929, **5**, 234) dyed material possesses a higher ash content than undyed material, and the ash content varies according to the country of origin; German yarns contain about 0.15%, whereas French rayons have, in general, the higher value of about 0.4%. Viscose rayons generally have ash contents of 0.20 to 0.35% on the air-dry weight, but some brands, *e. g.*, Dulenza and certain Staple fibres, such as Sniafil Seris and Châtelaine, have ash contents of about 0.75%. In the case of cuprammonium rayons, the variations are very great, ranging from about 0.15 to 1.50%. Cellulose nitrate silks with a few exceptions have ash contents greater than 1.0%, the amount generally being constant for individual brands. The following figures for ash contents of different brands of silk are taken from Reinthaler (*Artificial Silk*, 1928), but many of these refer to obsolete varieties.

	Ash %		Ash %
Cuprammonium Silks		Cellulose Nitrate Silks	
Hanau silk	0.32	Pearl silk	0.20
Hanau silk	0.51	Silkin silk	0.30
Hanau silk	0.73	Schwetzingen silk, 1923	0.65
Early Glanzstoff silk	1.50	Meteor artificial horsehair	0.90
Glanzstoff silk, 1913	0.37	Frankfurt-a-M. silk	1.00
Glanzstoff silk, 1913	0.16	Plauen silk	1.15
Berlin Glanzfaden silk, 1913	0.22	Jülich silk	1.30
Eagle silk, 1913	0.77	Tubize silk	1.30
Hölken silk, 1922	0.39	Sárvár silk	1.30
Sirius artificial horsehair	0.15	Obourg silk, extra filé, 1914	2.00
Excelsior artificial horse-hair	0.27	Ordinary Obourg silk, 1914	2.20
Brysilka, 1927	0.20	Obourg silk, 1927	0.36

Cellulose acetate rayon of the "Celanese" variety has the lowest ash content (0.10 to 0.15%) among commercial rayons, whilst in "Lustron" brands it exceeds 1.0%.

Ridge, Parsons and Corner (*loc. cit.*) have found values for ash of a similar magnitude to those given by Kami, but they believe that the amounts are, in general, so low that the determination has little practical value. According to these authors, Bemberg yarn (cuprammonium) contains less than 0.1%, acetate yarns have values of 0.09 to 0.16%, whilst other varieties contain about 0.35%. Certain undesulphurised viscoses and Obourg nitrocellulose rayon contain high percentages of ash (about 0.4%). The ash content, however, is very dependent on the conditions of bleaching and washing.

The alkalinity of the ash varies with the amount of ash, but if these two values are plotted against each other, the samples of rayon are found to fall into two groups: (*a*) a group where the alkalinity per grm. of ash is between 10 and 14, and (*b*) a group where this value is about 20 or more.

According to Herzog, a microscopical examination of the ash, if it has not been heated to too high a temperature, enables certain rayons to be differentiated from one another.

Acetic Acid Content.—The tendering of acetate silk most readily occurs when it is treated with hot alkaline liquids, owing to the loss of acetic acid caused by the hydrolysis of the cellulose acetate. The best method of determining the extent of the tendering is to estimate the acetic acid content. The amount of acetic acid found depends to some extent on the method of determination, but it should lie between about 53 and 54%, calculated on the dry weight of yarn.

There are two methods of determining the acetic acid content of acetate yarns. Ost's method (*Z. angew. Chem.*, 1919, **32**, 82) consists in treating 0.5 grm. of well cut up material, of known moisture content, with 5 c.c. of 50% (by volume) sulphuric acid for 20–24 hours at laboratory temperature. After dilution with water, the liquid is distilled in steam for at least 2 hours. After this period, each 50 c.c. of distillate should be tested until only one drop of 0.2 *N* sodium hydroxide solution is required to produce a red colour with phenolphthalein. The acetic acid in the distillate is titrated and expressed as grm. of acid per 100 grm. of dry acetate rayon.

Barnett's method (*J. Soc. Chem. Ind.*, 1921, **40**, 19) makes use of the solubility of the rayon in acetone, 1 grm. being dissolved and

10 c.c. of N sodium hydroxide added. The mixture should be shaken vigorously in order to prevent gel formation and then left for 24 hours. 10.5 c.c. of N sulphuric acid are then added and the mixture shaken for half an hour, after which the excess acid is titrated with 0.1 N sodium hydroxide. It is necessary to carry out a blank determination on the reagents. This latter method suffers from the defect that partly hydrolysed material is not soluble in acetone. In the Ost method it is difficult to know when to stop the distillation, since traces of acid may be found even after six hours.

Other Constituents. *Sulphur.*—Viscose yarns contain sulphur, which is formed in the chemical reaction which occurs when the alkali-treated cellulose is acted on by carbon disulphide in the preliminary stages of manufacture. Most viscose rayons are, however, treated with desulphurising agents, such as sulphides or ammonia, but this treatment never reduces the amount of sulphur to zero. The average value for sulphur content is about 0.03%, but undesulphurised viscoses may have values of between 0.2 and 0.4%. A comprehensive series of determinations of the sulphur contents of different types of rayon is given by Ridge, Parsons and Corner (*loc. cit.*). The values given by Wahl and Rolland (*loc. cit.*) are, however, on the high side.

Heavy Metals. *Copper.*—Contrary to expectation, the amounts of copper found in cuprammonium rayons is, in general, very small, and is smaller than those found in acetate and some Lilienfeld varieties. Owing to the catalytic effect of copper, it is probable that the presence of this metal is very undesirable.

Oils.—The amount of oil in the sample can be determined by extraction with ethyl ether in a Soxhlet extraction apparatus, using about 12 extractions in 2 hours. Dry-spun acetate rayon generally contains appreciable quantities of oil, the average value being about 3.0%.

Physical Properties

Microscopical Appearance.—The microscope is a valuable means of examining artificial silks and is of considerable assistance in recognising the different types of rayon. It must not be overlooked, however, that the microscopical characteristics of the fibres are to a great extent modified by the variable methods of manufacture by different makers. A full account of the technique is

given by Herzog (*Die mikroscopische Untersuchung der Seide und der Kunsteide*, 1924), while Bonnert (*J. Soc. Dyers and Col.*, 1922, **38**, 153) and King (*ibid*, 1927, **43**, 219) have described the microscopical characteristics of a number of samples of rayon.

The microscopical examination should consider the longitudinal form of the fibres, the shape of the cross sections and the average diameter of the filaments, although an estimation of the average cross-sectional area is a more accurate measure of the thickness. In order to determine the type of artificial silk, the diphenylamine test and staining with Indian ink and eosine (see below), in conjunction with the microscopical appearance of the fibres, is generally sufficient.

In examining artificial silk, however, the outline of the cross section is generally more characteristic of the fibre than the number and distinctness of the longitudinal striations, although the difficulties experienced in observing unsectioned fibres are due chiefly to optical effects, and under a rigidly controlled optical system must give identical results with those obtained by cutting transverse sections.

The thickness of the filaments of artificial silks is greater than that of natural silk. The determination of the average thickness is, however, only accurate when the filaments are approximately circular. According to Herzog, ordinary viscose rayons are about 30 to 35μ in diameter, although finer filaments are made in the U. S. A. Some nitrocellulose filaments are 16 to 20μ, but the finest ones are found in those cuprammonium and viscose rayons produced by the stretching process. Here the thickness is only 10 to 12μ. Except for spider silk, which is about 7μ thick, these filaments are finer than those of the natural silks, which vary from about 10 to 20μ. Acetate rayon fibres vary from 26.4 to 40.3μ. The uniformity of thickness is greatest in the case of cuprammonium, less so with acetate and viscose and least with nitrocellulose rayons.

Optical Behaviour.—Artificial silks are doubly refractive, and Herzog has made use of the degree of double refraction as a means of identification. Acetate rayon exhibits the weakest, and nitrocellulose rayon the strongest, specific double refraction. The strain in the fibres, which is the cause of the double refraction can, according to Faust and Littmann (*Cellulose Chem.*, 1926, **7**, 166), be measured by determining the shrinkage and extension of the fibres when immersed in water and in 4% sodium hydroxide solution.

Examination in Ultra-violet Light.—The application of ultra-violet light in distinguishing between different artificial silks has not yet been fully worked out; the results of different investigators appear to be at variance. To some extent, however, this method of examination enables certain brands of rayon to be identified. Bleached cuprammonium rayon made by stretch spinning from linters appears white with a strong bluish-violet fluorescence, whilst a Brysilka rayon made from sulphite cellulose, which was bleached and sized, was yellowish-white with a faint violet fluorescence when observed in ultra-violet light. Sizing, however, appears to affect the intensity of the fluorescent light. Cellulose acetate rayons fluoresce more brightly than other kinds. Raw natural silk, after degreasing, appears white with a strong blue fluorescence. Some viscose rayons have given a yellow fluorescence, although the majority appear yellow or white with a bluish-violet fluorescence; on the whole, this method of examination does not appear to be very satisfactory.

Examination in X-rays.—Herzog has applied X-rays to the differentiation of the different varieties of artificial silk, but for further information reference should be made to his book (*loc. cit.*). The application of X-ray analysis in determining the structure of artificial silk is described by Clark (*Ind. Eng. Chem.*, 1930, **22**, 474).

Breaking Strain and Elongation.—The relative strength of the fibres in the dry and wet state is often a certain test of their origin. Ordinary silk retains its full strength on wetting, but the cellulose products may lose 50% of their strength on wetting in cold water for 5 minutes. Care must be taken when making this test, as the full strength is regained on drying. According to Lüdke (*Kolloid Z.*, 1929, **47**, 341), however, the tenacity of fibre is affected by the surface tension of the liquid in which the silk is immersed, liquids of low surface tension increasing the strength.

The actual strength of the fibres in relation to their size is a valuable indication as to their origin, but different investigators seem to obtain varying results probably owing to the different methods used for testing. The ordinary silk serimeter should be used with automatic attachment (See Dreaper, *J. Soc. Chem. Ind.*, 1909, **28**, 1300; and Dreaper and Davis, *ibid.*, 1912, **31**, 100). In carrying out any determinations of tensile strength, the humidity of the

atmosphere must be rigidly controlled. The extent of the variations in strength with humidity is indicated by King and Johnson (*J. Soc. Dyers and Col.*, 1928, **44**, 346) who have published data showing the variations which are found in the tensile strength as the humidity is altered. Wolfgang (*Kunsteide*, 1926, **8**, 175) attributes brittleness of the fibres to traces of acid.

Identification.—The identification of the different types of artificial silk is carried out by means of the following tests.

Burning Test.—Silk burns slowly with a smell resembling burnt horn. Weighted silk burns and leaves a skeleton thread of mineral matter. Nitrocellulose, viscose and cuprammonium products burn quickly, like cotton. Cellulose acetate filaments burn very much like real silk, in that they melt and form charred globules at the end of the unburned part of the threads. Casein and gelatin products burn like silk. Care must be taken, however, in employing this test, since composite threads of silk and rayon are on the market.

Diphenylamine Reaction.—This reaction is the most reliable means for differentiating between the three main types of artificial silks. It is based on the fact that all cellulose nitrate silks, even though they have been denitrated, contain traces of nitric acid. A small quantity of diphenylamine is added to concentrated sulphuric acid and shaken until dissolved in a glass-stoppered flask fitted with a glass rod. This reagent is spotted on the fabric to be examined. The threads swell and, in the case of nitrocellulose rayon, a deep blue colour develops. A yellow to brown coloration is given with cuprammonium and viscose artificial silks. A mixture of two parts of sulphuric acid to one part of glacial acetic acid, is, however, recommended as preferable to sulphuric acid alone, since artificial silk does not dissolve so quickly in this mixture and the blue colour persists for a longer period of time. The reagent, however, does not keep indefinitely and should be discarded when it becomes dark in colour.

In the case of dyed material, the dye may be bleached with calcium or sodium hypochlorite solution to which hydrochloric acid has been added, or by means of a solution of an alkaline hydrosulphite.

Microscopical Appearance.—*Cuprammonium rayon* is cylindrical. Circular cross sections with smooth edges may also be found in the case of viscose rayon if the coagulating bath contains mineral acid without the addition of salts, but this type is very unusual. The

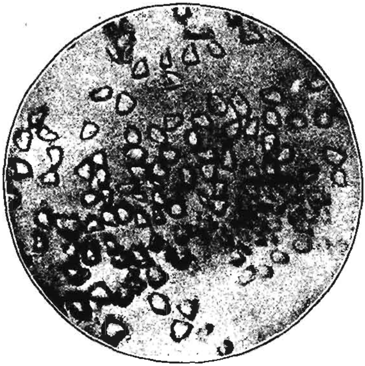

FIG. 7—Silk. × 200.

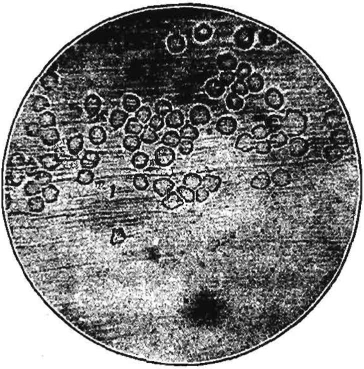

FIG. 8.—Cuprammonium. × 260.

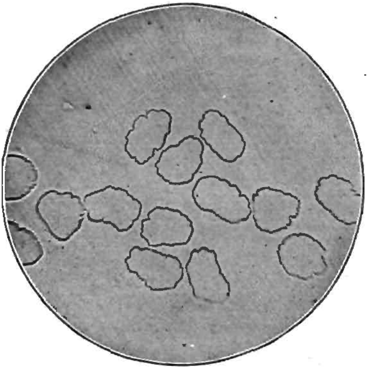

FIG. 9.—Obourg Viscose. × 260.

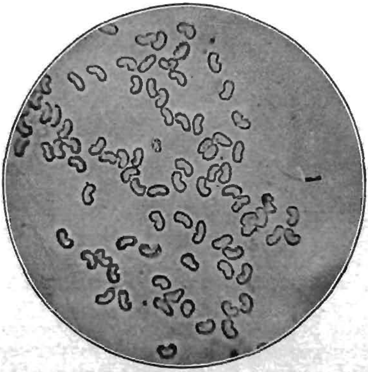

FIG. 10.—I. G. Viscose. × 260.

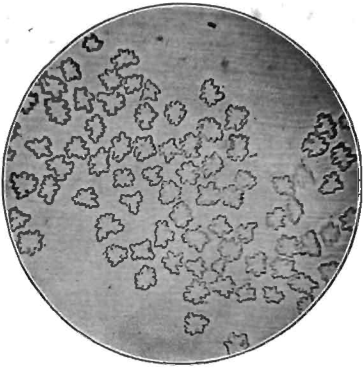

FIG. 11.—Courtauld's Tudenza (Viscose). × 260.

FIG. 12.—Obourg Nitro. × 260.

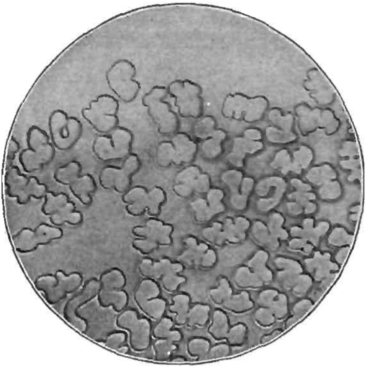

FIG. 13.—Courtauld's Seraceta (Acetate). × 260.

FIG. 14.—Mercerised Cotton. × 260.

addition of ammonium sulphate to the coagulating bath produces non-angular, irregular-shaped cross sections. Cuprammonium rayon filaments are almost equal in thickness along their length with occasionally fine longitudinal striations. Herzog states (*loc. cit.*) that cuprammonium rayons can be distinguished from a viscose rayon with circular cross sections by means of the ultramicroscope, the former possessing a transverse network.

Viscose rayon shows a large number of fine longitudinal striations, together with a few coarser ones. The cross section is irregular and often serrated. Some varieties, however, do not show the longitudinal striations, and the shape of the cross section must be used in order to differentiate between such viscose rayons and cuprammonium artificial silk.

Nitrocellulose rayons are characterised by the presence of a few but pronounced longitudinal striations in the fibres, while many types possess a flat, ribbon-like appearance. Wet-spun nitrocellulose rayons possess a greater number of longitudinal striations than do dry spun rayons. The cross section is generally irregular in shape, but with a smooth outline.

Cellulose acetate rayons vary to some extent in their microscopical characteristics, depending on the particular method of manufacture, but the variations are not so great as in the case of viscose rayons. Dry-spun acetate rayon is similar in appearance to dry-spun nitrate rayons. It does not exhibit the fine striations found in the viscose types. Acetate rayon, however, can easily be recognised by its solubility in acetone, glacial acetic acid, etc.

Differentiation of Cuprammonium and Viscose Rayons.—The differentiation of some viscose rayons from those of the cuprammonium variety can be carried out by the following test, due to Reinthaler, which has been found to give reliable results: A small sample of the rayon is dyed in a mixture of 15 c.c. of Pelican Ink, No. 4001 (Günther Wägner), 20 c.c. of a 0.5% solution of eosine extra (I. G.), and 65 c.c. water for 5 minutes at room temperature. The sample is then thoroughly rinsed and dried at a temperature not exceeding 60°. Cuprammonium rayon made by the stretch spinning process is coloured deep blue, whereas viscose artificial silk is coloured light bluish-red.

A further test which, however, is not perfectly reliable can be carried out by boiling the sample with Nessler's reagent and then

rinsing with a solution of potassium iodide. Cuprammonium rayons are unstained, whilst the viscose varieties are coloured grey to orange according to the amount of sulphur present. It is, however, unsafe to judge on such a test alone, and the result must be confirmed by using other tests. Weltzien suggests that the silks should be treated at 18–19° with 9% sodium hydroxide solution. Nitro-silk dissolves, viscose rayon loses about 40–50% of its weight, whilst cuprammonium rayons only lose 30% of their weight in 30 minutes.

Schwalbe (*Färb. Zeit.*, 1907, **18**, 237), however, recommends a qualitative test in which about 0.2 grm. of the artificial silk is warmed on a water bath with 4 c.c. of Fehling's solution for about 10 minutes and water then added. If the liquid retains its blue colour, the sample is cuprammonium rayon; but if the liquid becomes green, viscose or nitrate rayon is indicated.

Other methods, depending on the reducing power of the rayon, include the use of ammoniacal solutions of silver oxide (Götze, *Textilber.*, 1925, **6**, 759).

The application of a solution of iodine and potassium iodide in zinc chloride solution colours viscose rayon bluish-green, and leaves cuprammonium rayon colourless. There are, however, many exceptions to this test.

Detection of Unaltered Nitrocellulose.—Dimethylaniline is claimed by Walter (*Z. angew. Chem.*, 1911, **24**, 62) as a reagent for detecting any unaltered nitrocellulose in artificial silk prepared from that product. The material is immersed in the dimethylaniline and exposed in porcelain basins to the action of the air in the dark at 13–15°. The colour-change to a greenish tint after 24 hours, changing to intense bluish-violet at 60 hours, and violet at 156 hours, was noticed with nitrocellulose itself or celluloid.

Loss of Weight on Boiling with Alkali.—When rayons are boiled in a 1% solution of sodium hydroxide, they lose a certain amount of weight, and this loss is approximately proportional to the copper number. The conditions under which this test should be carried out are described by Clippens, Geake and Ridge (*J. Text. Inst.*, 1927, **18**, T277).

Variations in Swelling Power.—The swelling power of artificial silks in 10% sodium hydroxide solution can be used as a means of differentiating the different types. Thus Weltzien (*Textilber.*, 1926, **7**, 338; cf., Lowrie, *J. Soc. Dyers and Col.*, 1928, **44**, 76) recommends

that lengths of 50 cm. of the silk be extended by a small weight (4 mg.) and dipped in the sodium hydroxide solution. The increase in length is greater with viscose than with cuprammonium rayon, some samples of cuprammonium even contracting when immersed in such a solution. If the threads are then placed in a cylinder of water, viscose rayon will shrink, whereas cuprammonium rayon increases in length.

Action of Cellulase.—Karrer (*Helv. Chim. Acta*, 1925, **8**, 797; 1927, **10**, 430; 1928, **11**, 231) makes use of the enzyme, cellulase, for investigating the effect of different methods of manufacture on the properties of the silk. Stretching viscose threads during manufacture decreases the amount of enzymic hydrolysis. The composition of the coagulating bath also affects the ease with which this enzyme can break down the cellulose.

In making use of chemical tests it must be realised that the tests have been elaborated with a view to determining how far the cellulose has been affected by the process of manufacture, but it is obvious that the condition of the starting material as well as the presence of impurities may affect the results. The results of any determination must, therefore, be treated with some small amount of reserve.

Examination of Mixtures.—The amounts of different materials in a mixture are determined by making use of the solubility of acetate silk in acetone. Regenerated celluloses are soluble in cuprammonium, whilst wool and silk are insoluble. Lloyd and Priestly (*J. Soc. Dyers and Col.*, 1929, **45**, 201) hydrolyse the sample for 20 minutes at 50° with 30% (by volume) sulphuric acid. Cotton and wool are unaffected by this treatment. The wool can be separated from the cotton by allowing the acid to act for 24 hours at 25°.

Artificial Silk Standards.—Artificial silks, with the exception of the acetate variety, vary in weight considerably with the humidity of the atmosphere, and, for purposes of commerce, a series of regains or water allowances has been drawn up. The Bureau International pour la Standardisation des Fibres Artificielles (B. I. S. F. A.) was founded in 1928 to fix standards, classifications and denominations of the different categories of artificial textile fibres. According to the 1931 edition of their booklet, the moisture regain for viscose and cuprammonium yarns has been fixed at 11%, although it is realised that under normal atmospheric conditions these two types of rayon

may absorb more than 12% of water. Acetate yarns absorb less moisture, and the regain allowed has been fixed at 5.5%, to which is added 3.0% to represent the average percentage of oil which is usually found in this variety of yarn. This oil content, however, is subject to variations.

According to the B. I. S. F. A., a viscose yarn is considered to have undergone special treatment if it contains besides moisture more than 2% of extractable matter. In determining the Yarn number, tenacity, extension and twist, the yarn must be in the form of skeins and the skeins must be made without altering the twist. The skeins must be suspended for 24 hours at a temperature of *20° and a relative humidity of 60% (a tolerance of ±2 for each* of these values is permissible), the air being in movement, before any determination of the denier, tenacity, extension or twist is attempted.

The Yarn number is expressed in deniers, *i. e.*, the weight in grams of 9,000 metres. Twenty determinations should be made, and the variations in weight between the results must not exceed 7% for the lighter yarns, and 4% for the heavier ones in the case of "A" (first) quality.

The tenacity or breaking load expressed in grm. per denier must be determined by a strength tester of the pendulum type and given to the second decimal place, the last figure being either 0 or 5, in order to approach as closely as possible to the figure found. The extension at the breaking point is to be expressed as a percentage on the original length.

The twist is expressed as turns per meter of length, and is determined by completely untwisting lengths of 50 cm. When the thread is held vertically at the level of the eye, the spirals are seen with a downward inclination to the right if the twist is "right," and *vice-versa* for a left twist.

The selling weight of the yarn is determined after conditioning by drying 250 grm., at 105° in a conditioning apparatus until the loss in weight does not exceed 0.05% on drying for a further 10 minutes. The difference between the moisture contents of two duplicate samples should not exceed 1%. Similar conditions apply in the case of cuprammonium yarns.

In the case of dry-spun acetate rayon, the skeins must be degreased before conditioning. The degreasing may be carried out either by

means of ethyl ether in a Soxhlet extraction apparatus, 12 extractions in about 2 hours being required, and the skeins dried in a ventilated oven at 105° for 30 minutes, or grease may be removed by means of "Lux" soap, the procedure being, as follows:

1st bath—15 minutes in a 0.5% solution of "Lux" in distilled water at 50° C.

2nd bath—5 minutes in distilled water at 50° C.

3rd bath—15 minutes in a 0.1% solution of "Lux" in distilled water at 50° C.

4th, 5th and 6th baths—5 minutes in distilled water at 50° C.

The bulk of water is then removed by centrifuge and the skeins dried in a well ventilated oven at 105°.

In considering any analytical data concerning artificial silk, it must be remembered that this industry is a young and modern one, and that progress is being made at such a rate that the data obtained only a few years ago cannot be safely applied to the latest varieties of rayon. It is essential, therefore, that the analyst should refer to the most recent work before forming conclusions as to the interpretation to be placed on his analysis.

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The writer extends his thanks to Dr. H. Hunter, for reading through this section, for his very helpful criticism, and for kindly supplying the photomicrographs of different types of rayon which are used in illustrating the text.

CHITINOIDS

The substance known as chitin occurs throughout the invertebrata in the form of an investment to the outermost cellular layer or ectoderm. In many cases, a chitinous composition has been ascribed to structures solely on account of their insolubility in alkali hydroxides and in dilute acids, or even in only one of these reagents. At least two distinct compounds, chitin and conchiolin, have been confounded in this manner. They exhibit essential differences in characters and composition, and, together with spongin, may be

conveniently classed together as "chitinoids." The following table shows the ultimate composition attributed to these three substances:

	Chitin	Conchiolin	Spongin
Carbon	46.32	50.7	47.44
Hydrogen	6.40	6.5	6.30
Nitrogen	6.14	16.7	16.15
Oxygen	41.14	26.1	30.11
	100.00	100.00	100.00

Chitin ($C_{10}H_{30}O_{12}N_2$ or $C_{30}H_{50}O_{19}N_4$)

Chitin, as it occurs in nature, is frequently impregnated with calcareous matter, as in the shells of the crustacea, or with silica, as in the radula of the higher mollusca.

Chitin was prepared by Hoppe-Seyler by boiling the wing-cases of the cockchafer with dilute solution of sodium hydroxide until they became colourless. The product was then boiled in succession with water and dilute hydrochloric acid, and finally with boiling alcohol and with ether.

Chitin may be obtained by a similar process from the shell of the crab or lobster, but in this case the substance should be previously digested with hydrochloric acid, to dissolve the earthy matters deposited in the chitinous tissue.

Chitin is a colourless, amorphous substance, which, when prepared in the foregoing manner, retains the form of the parts composed of it. It is insoluble in water, alcohol, ether, acetic acid, and in dilute mineral acids. Chitin resists in a remarkable manner the action of alkalies, and can be subjected to a prolonged treatment with their boiling concentrated solutions without undergoing decomposition.

Chitin is dissolved by strong mineral acids. If the chitin prepared as above described is treated with cold, concentrated hydrochloric acid, and the solution diluted with a large excess of water, the chitin is precipitated in a colourless, gelatinous form.[1]

[1] C. F. W. Krukenberg (*Z. Biol.*, 1886, **22**, 480) finds that the action of cold hydrochloric acid on chitin for 1 hour is not one of simple solution. A chlorinated compound is first formed, which swells up in the acid, and on filtering, a cloudy filtrate is obtained, from which about 2 % of chitin is precipitated by water or baryta. The filtrate from this precipitate does not contain glycosamine or other decomposition products. After longer action of hydrochloric acid, the chlorinated substance suffers partial or complete disintegration, and the filtered liquid contains a dextrinoid substance of feeble reducing power, together

The composition of chitin has been investigated by Ledderhose, under the direction of Hoppe-Seyler and Baumann (*Z. physiol. Chem.*, 1878, **2**, 213), who, as the mean result of twelve analyses, found it to contain: Carbon, 45.69; hydrogen, 6.42; nitrogen, 7.00; and oxygen, 40.89%. Analytical results by many investigators will be found in the *Biochemische Lexikon*. (Vol. **2**, 191, 627).

Frankel and Kelly (*Monatsh.*, **23**, 132) find that chitin undergoes hydrolysis when heated with acids, with formation of glucosamine and acetic acid, according to the following equation:

$$C_{18}H_{30}O_{12}N_2 + 4H_2O = 2C_6H_{13}O_5N + 3C_2H_4O_2$$

Irvine (*J. Chem. Soc.* 1909, **95**, 564) considers that 3 molecules of acetyl-amino-glucose and 1 of amino-glucose take part in the condensation, with loss of 4 molecules of water. Brach (*Biochem. Z*, 1912, **38**, 468) considers that 4 molecules of mono-acetyl glucosamine are condensed, giving a formula $C_{32}H_{54}O_{21}N_4$.

When chitin is boiled with hydrochloric acid the hydrochloride of glucosamine is formed. This is soluble in water, crystalline, and dextrorotatory, $[\alpha]_D 70.6°$. The sulphate crystallises in needles. Enoch Zander (*Pflüger's Arch.*, **66**, 545) gives a microchemical test for chitin. The substance is treated with a drop of strong potassium iodide solution and this is followed by conc. zinc chloride. The cell structure takes a violet colour and the chitin a brown one.

Conchiolin ($C_{30}H_{48}O_{11}N_9$)

This substance is obtained by macerating the shells of mussels or snails in dilute hydrochloric acid, and then boiling with sodium hydroxide solution.

Conchiolin closely resembles chitin, with which substance it was formerly supposed to be identical. It is distinguished from chitin by its larger content of nitrogen, and by yielding leucine when boiled with dilute sulphuric acid, without any sugar-like substance being simultaneously formed.

Conchiolin gives the biuret, Millon, and xanthoproteic reactions, but does not react with lead sulphide.

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with very small quantities of chitin (precipitable by water) and glucosamine hydrochloride. By the action of 5 and 10% solutions of potassium or sodium carbonate saturated with chlorine, chitin was converted into a substance corresponding with amidulin. After 12 days a small quantity of chitin had dissolved, and after filtering and removing the salts by dialysis a substance was obtained which dissolved readily in cold water, reduced Fehling's solution on heating, and gave, both with neutral and with basic lead acetate, copious precipitates insoluble in excess of the reagent. These characters and its indiffusibility distinguish the substance from glucosamine hydrochloride.

Spongin

Spongin, or spongiin, is the characteristic protein substance of sponges.[1] It has been compared to the collagens and to the fibroin of silk, but, on the whole, appears to be best classed with the chitinoïds.

Spongin gives positive reactions with protein reagents with the exception of those of Millon and Adamkiewicz. Spongin contains 1.5% of iodine present largely as diiodotyrosin (Ostwald, *Z. Phys. Chem.*, 1911, **70**, 310; **71**, 200; **72**, 374; 12, **74**, 290; **75**, 353).

Spongin is obtained when sponge is boiled in succession with dilute hydrochloric acid, dilute sodium hydroxide, water, alcohol, and ether. The product contains 16.15% of nitrogen.

Spongin is unaffected by the reagents employed for its preparation, and is also insoluble in ammonia. It is dissolved slowly by strong alkalies, and is reprecipitated on neutralising the solution. Dilute acids have no action on spongin in the cold, but it is dissolved by concentrated acids.

When boiled with water under pressure, spongin yields no gelatin. Boiling dilute sulphuric acid is stated by Städeler (*Annal. Chem. Pharm.*, **111**, 12) to decompose it with formation of leucine and glycine, but not tyrosine.[1] Kossel and Kutschen (*Z. physiol. Chem.*, **31**, 165) obtained arginine, lysine and glutamic acid on hydrolysis with sulphuric acid. V. J. Clancey (*Biochem. J.*, 1926, **20**, 1186) examined the spongin from the ordinary bath sponge (*Hippospongia equina*) and found that glutamic acid and glycine were the chief products of hydrolysis. Leucine, aspartic acid, arginine, proline, lysine, tryptophane and iodogorgionic acid were also present in the hydrolysate, and he considers that spongin is more allied to the collagens and fibroin of silk than to the chitinoids.

Spongin yields on digestion peptone-like substances which, however, do not give the usual colour reactions (xanthoproteic, Millon's or Adamkiewicz tests).

Spongosterol, ($C_{19}H_{32}O$) has been isolated from an ethereal solution of *Suberites domuncula*, with a m. p. of 119–120° and giving $[\alpha]_D$ =

[1] An interesting description of the natural history of sponges has been published by Holmes (*Pharm. J.*, [iii], **17**, 991). Information on the methods of *bleaching* sponges will be found in the *Pharmaceutical Journal*, [iii], **14**, 88, and in the *Chemist and Druggist*, **30**, 643. Potassium permanganate is commonly employed. According to a recipe in *Le Moniteur de la Teinture.* (abst. *J. Soc. Dyers and Col.*, **4**, 63), sponges may be conveniently bleached by immersing them in saturated bromine water for several hours. This treatment is repeated till the desired tint is obtained. The sponges are then passed through a bath of dilute sulphuric acid, and finally washed with cold water. It is stated that the treatment in no way injures the quality of the sponge.

19.59°. Its crystals give no coloration with 20% sulphuric acid (Henze, *Z. physiol. Chem.*, 1904, **41**, 108).

Cornin forms the framework of corals, and yields indole.

KERATIN SUBSTANCES. KERATOIDS

When cuticular and allied tissues—including nails, horns, hoofs, feathers, whalebone, scales, etc.—are treated in succession with boiling ether, alcohol, water, and dilute acids, the insoluble residue retains the shape of the original tissue and is known as keratin. The product varies somewhat in its characters and composition with its origin, so that a number of allied substances are comprehended under the general name of keratin. The following table illustrates the composition of the "keratin" obtained from different sources:

Source of keratin	Carbon	Hydrogen	Nitrogen	Sulphur	Oxygen (by difference)	Authority
Feathers	*52.46*	*6.94*	*17.74*	?	*22.86*	....
Quills	51.7	7.2	17.9	....	....	Scherer.
Wool[1]	50.65	7.03	17.71	4.61	20.00	Scherer.
Hair (man's)[1]	50.65	6.36	17.14	5.00	20.85	Van Laer.
Fur (white rabbit's)	49.45	6.52	16.81	4.02	23.20	Kühne and Chittenden.
Nails	51.00	6.94	17.51	2.80	21.75	Mülder.
Nails	51.09	6.82	16.90	2.80	22.39	Scherer.
Horn (cow's)	51.03	6.80	16.24	3.42	22.51	Tilanus.
Hoof (horse's)	51.41	6.96	17.46	4.23	19.94	Mülder.
Epithelium	51.53	7.03	16.64	2.18	22.32	Hoppe-Seyler.
Epidermis	50.28	6.76	17.21	0.74	25.01	Hoppe-Seyler.
Whalebone[2]	51.86	6.87	15.70	3.60	21.97	Van Kerckhoff.
Tortoise-shell	54.89	6.56	16.77	2.22	19.56	Mülder.
Neuro-keratin (brain)	56.99	7.53	13.15	1.87	20.46	Kuhne and Chittenden.

[1] Other analyses of the keratin from wool are given below.

[2] Substitutes for whalebone have been protected by various inventors including the following English patents: J. Baier, 1895, No. 10193; W. Hunkemöller, 1896, No. 1214; and Martin & Levy, 1895, No. 1820; 1896, No. 4718.

Bourquelot (*Pharm. J.*, [iii], **19**, 1035) gives the following limits of composition of keratoids: Carbon, 50.3 to 52.5; hydrogen, 6.4 to 7.0; nitrogen, 16.2 to 17.7; oxygen, 20.7 to 25.0; and sulphur, 0.7 to 5.0%.

The results shown on p. 852 have been given by Abderhalden and Fuchs (*Z. physiol. Chem.*, 1908, **57**, 339) for keratin from 4-year old animals.

It appears that, while the keratin from different sources is tolerably constant in the proportions of carbon and hydrogen contained in it, the percentages of sulphur and of oxygen vary within limits. This

	Hoof of ox	Horn of ox	Hoof of horse
Dry residue	91.5 %	96.5 %	75.4 %
Ash	0.16%	0.36%	0.45%
Melanin substance	0.12%	0.96%	0.9 %
Glutamic acid hydrochloride in ash, dry residue	16.8 %	12.9 %	18.2 %

fact is further illustrated by the following results recorded by Mohr (*Z. physiol. Chem.*, 1895, **20**, 403).

Source of keratin	Sulphur, %	Source of keratin	Sulphur, %
Woman's hair; dark blonde	4.95	Pig's hair	3.59
Girl's hair; dark brown	5.34	Sheep's wool	3.68
Boy's hair; red blonde	4.98	Goose feathers	2.59–3.16
Boy's hair; red	5.32	Pig's hoof	2.69
Rabbit's hair	4.01	Calf's hoof	3.57
Calf's hair	4.35	Ox hoof; white	3.49
Horse's hair	3.56	Ox hoof; black	3.45

All the keratoids yield more or less ash on ignition, the proportion in some cases being considerable. The nature of the ash varies with the source of the keratin. Thus hoofs and horns given an ash consisting chiefly of calcium phosphate, whilst the ash of wool is largely composed of potassium and sodium sulphates. The ash of hair consists chiefly of sulphates of alkali metals, ferric oxide, and silica. The silica in the ash of feathers is stated by von Bibra to range from 27 to 40%.

The greater part of the sulphur of keratoids is only loosely combined, so that on boiling hair, wool, or feathers with lead acetate and excess of sodium hydroxide, the liquid blackens from formation of lead sulphide. Hoppe-Seyler found that by heating horny substances with bartya water in sealed glass-tubes, nearly the whole of the sulphur is obtained as barium hydrosulphide.

When heated in the dry state, keratoids swell up, char, and evolve a characteristic odour of burnt feathers.

Many of the keratoids are very hygroscopic, taking up a large proportion of water without affording any indication of its presence beyond the increase in the weight of the substance.

The keratoids are quite insoluble in alcohol or ether, and are not much affected by boiling with water at the ordinary pressure; but, when heated under pressure for a long time at 150 to 200°, they dissolve with evolution of hydrogen sulphide, to form a turbid solution which does not gelatinise on cooling, and gives, on evaporation, a residue insoluble in water.

Keratin yields aspartic acid, leucine, and tyrosine on boilirg with dilute sulphuric acid. Gümbel gives the following values for its nitrogen content: 1.17 as amide-N., 0.42 as melanin-N., 2.95 as diamino-N., and 11.51 as monamino-N. It yields no plastin or coagulose on digestion, and is the least soluble of the albuminoids, (Smith, *Z. wissensch. Zool.*, 1883, **19**, 469). Successive treatment with acid, alkali, pepsin, and trypsin will remove all other albuminoids, (Kühne and Chittenden, *Z. wissensch. Zool.*, 1890, **26**, 291). It is remarkable for its high percentage of tyrosine and cystine and gives intense Millon's, lead sulphide, xanthoproteic and Adamkiewicz reactions.

The effect of different alkalies on keratoids is characteristic of these substances. Caustic alkalies in the cold cause them to swell and eventually to dissolve. Ammonia has a somewhat similar action. Suspensions of lime do not bring about much swelling of the keratoid, but are able to bring about the disintegration of the material much more readily than dilute solutions of sodium or potassium hydroxides. This dissolution of the protein is apparently connected with an initial hydrolysis of the disulphide linkage (—S—S—) of the cystine portion of the keratin molecule. When the reaction occurs under such conditions that reduction of the sulphur to sulphydryl can occur, the breakdown of the protein becomes very rapid. It appears that suspensions of lime are more effective in producing reducing substances from the keratin than are other alkalies (Marriott, *J. Soc. Leather Trades' Chem.*, 1925, **9**, 599; 1928, **12**, 216). The addition of strong reducing agents, such as sodium sulphide, potassium cyanide or stannous chloride, to an alkaline solution accelerates the breakdown to a very great extent, provided the *p*H value of the solution is greater than 11.

When hair is digested with 2 *N* sodium hydroxide for 18 hours at 25°, most of the hair dissolves. If this liquor is filtered and then brought to *p*H 4, a voluminous white precipitate is formed which has been called "keratose" (Wilson and Merrill, *J. Amer. Leather*

Chem. Assoc., 1926, **21**, 2). This material is digested by pancreatin according to Wilson, but Starey (*Z. physiol. Chem.*, 1925, **144**, 147) states that trypsin is without effect (see also Langecker (*Z. physiol. Chem.*, 1919, **108**, 230).

When treated with cold glacial acetic acid, horny substances swell up, and, on boiling, are largely dissolved. Whalebone is converted into a gelatinous substance by boiling with concentrated acetic acid, but tortoise-shell is little changed by such treatment.

Nitric acid turns keratoid bodies yellow, and, on application of heat, dissolves them, with formation of oxalic acid and other products.

On treatment with cold concentrated sulphuric acid, the keratoids swell up, and, on heating, dissolve more or less completely. The solution appears to contain syntonin, for on dilution with water and addition of potassium ferrocyanide it yields a flocculent precipitate, and also gives a white flocculent precipitate when exactly neutralised.

When chlorine is passed into water containing a finely divided keratoid, or when a keratoid is treated with bromine water, the substance undergoes no change in external appearance, but after drying it is harsh to the touch, and then dissolves in ammonia, with evolution of nitrogen. According to Stary (*loc. cit.*) bromine in glacial acetic acid or hydrogen peroxide in 4.*N* sulphuric acid renders the protein soluble in alkali and easily hydrolysable by trypsin.

When treated with fuming hydrochloric acid, most keratoid bodies swell up to a jelly and subsequently dissolve, with the exception of hair, which is unaffected by such treatment.

Keratin is not acted on either by pepsin or trypsin, but the keratin of the Malpighian layer of skin after treatment with 0.1 *N*. caustic soda for about 18 hours in the cold is rapidly digested by trypsin (Rohm, *Collegium*, **519**, 374 (1913).

When keratoid substances are boiled with dilute sulphuric acid, they undergo decomposition with formation of aspartic acid, volatile fatty acids (including propionic acid), ammonia, leucine, and from 3.5 to 4.0% of tyrosine. The formation of the last substance distinguishes keratoids from the collagens. Keratoids also differ from the collagens in not yielding gelatin by the action of superheated water or dilute acids, and in containing a notable proportion of sulphur.

The more recent examinations of the different forms of keratin have been concerned with the amounts of the different amino acids

	Source of Keratin	Cys-tine	Gly-cine	Ala-nine	Leu-cine	Glu-tamic Acid	Phen-ylal-anine	Argin-ine	Tyro-sine	Val-ine	Pro-line	Ser-ine	Ly-sine	Aspar-tic Acid	Histi-dine	
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1.	White human hair	11.55	9.12	6.88	12.12	8.0	0.62	......	3.3	....	....	...	....	......	......	..
	Scales of *Manis Japonica*	.....	1.33	12.0	10.25	3.5	2.67	......	13.0	4.0	3.5	....	....	......	......	..
2.	Elephant-epidermis	4.70	8.33	5.07	3.6	10.2	2.33	......	5.20	2.43	....	....	....	......	......	..
3.	Cow horn	.....	0.34	1.2	18.3	3.0	3.0	......	......	5.7	.....	0.68	....	2.5	......	..
4.	Sheep wool	7.3	0.58	4.4	11.5	12.9	....	......	2.0	2.8	4.4	0.1	....	2.3	......	..
	Ram's horn	7.5	0.45	1.6	15.3	17.2	1.9	2.7	3.6	4.5	3.7	1.1	0.2	2.5	......	..
5.	Horsehair	7.9	4.7	1.5	7.1	3.7	0	4.45	3.2	0.9	3.4	0.6	1.1	0.3	0.6	.
6.	Goose feathers	.....	2.6	1.8	8.0	2.3	....	......	3.6	0.5	3.5	0.4	....	1.1	......	..

1. Buchtala, *Z. physiol. Chem.*, 1913, **85**, 241, 246, 335.
2. Buchtala, *Z. physiol. Chem.*, 1912, **78**, 55.
3. Fischer and Dorpinghaus, *Z. physiol. Chem.*, 1903, **36**, 462.
4. Abderhalden and Voitinovici, *Z. physiol. Chem.*, 1907, **52**, 348.
5. Abderhalden and Wells, *Z. physiol. Chem.*, 1905, **46**, 31.
6. Abderhalden and Le Count, *Z. physiol. Chem.*, 1905, **46**, 40.

which can be obtained after hydrolysis with acid. Some analyses are given in the table, but the reader is referred to Oppenheimer (*Handbuch der Biochemie*, Section III, by Strauss and Collier, 1924).

Other workers have examined keratin for certain amino acids. Thus Abderhalden and Fuchs (*Z. physiol. Chem.*, 1908, **57**, 339) have determined the glutamic acid content of various keratins from horns and hoofs and find variations between 13 and 18%.

The cystine content of hair, wool and feathers has been determined by Wilson and Lewis (*J. Biol. Chem.*, 1927, **73**, 543), who have found that the amounts present are very variable. Their results are given below.

Human hair	15.6 to 21.2%
Sheep wool	8.0 to 10.9
Feathers	7.0 to 12.2
Rabbit hair	11.9 to 14.0
Rat hair	14.1
Dog hair	19.0

They find that there is no relationship between the colour of the hair, age or sex of the animal and the cystine content. They also state that there is little evidence of non-cystine sulphur, a finding which is confirmed by the later work of Rimington (*Biochem. J.*, 1929, **23**, 41) on wool. Rimington considers that cystine is best determined by means of Sullivan's method, using sodium β-naphthoquinone sulphonate (*U. S. Public Health Rep.*, 1926, **41**, 1030).

The chemical constitution of keratin is as yet undecided, but it seems likely that it contains a disulphide linkage which is probably masked by an anhydrous linkage (Speakman, *J. Soc. Chem. Ind.*, 1931, **50**, 6T). This linkage can be broken by alkalies, and especially alkaline reducing agents, and the characteristic resistance of keratin to ordinary reagents is then reduced. According to Abderhalden and Vortinovici (*loc. cit.*), however, keratin is a mixture of proteins, and the variations in the amounts of the amino acids tend to confirm this supposition. Unna (1920, Abs. *J. Chem. Soc.*, 1921, *i*, 637) has examined horn and epidermis and states that there are three forms of keratin: "A" which gives the xanthoproteic reaction and is insoluble in fuming nitric acid; "B" which is yellow and dissolves in fuming nitric acid; "C" which is yellow and insoluble in fuming nitric acid.

Stather (*Gerber*, 1927, **53**, 9) has attacked the problem of the constitution of keratin by examining various peptides containing cystine in order to find out which of them yield sulphide sulphur when treated with dilute sodium hydroxide, on the assumption that only those peptides which yield sulphide sulphur are likely to be present in keratin. He finds that dialanyl-cystine-dihydrin on decomposition by 0.1 *N* sodium hydroxide yields 71% of its sulphur as sulphide or polysulphide. It is interesting to note that cystine itself is not decomposed in this way by dilute sodium hydroxide (Marriott, *loc. cit.*).

Langecker (*loc. cit.*) has digested horn with solutions of sodium hydroxide at 40° and separated the protalbumoses by means of sodium chloride. On the addition of acid, deuterokeratose is precipitated which, he states, is resistant to proteolytic enzymes.

A very good summary of the literature of keratin is given by Barrett (*J. Text. Inst.*, 1926, **17T** 111).

"Keratin" is official in the German Pharmacopœia (3rd edition).[1] It is directed to be prepared by digesting shavings of quills with a mixture of equal parts of ether and spirit, and subsequently with an acidified solution of pepsin at 40°. The residue is dissolved in acetic acid by prolonged boiling (30 hours), the solution strained, evaporated to a syrup, and dried on plates. The product forms a brownish-yellow, tasteless, and odourless powder or scales. It should not yield anything to water, spirit, ether, dilute acids, or pepsin-hydrochloric acid; and should not yield more than 1% of ash, nor contain more than 3% of matter insoluble in acetic acid or ammonia.

Small quantities of azelic acid (0.046 grm. from 250 grm.) are formed when this substance is oxidised with potassium permanganate. Similar results have been obtained from horn after extraction with ether to remove grease. (Lissizin, *Z. physiol. Chem.*, 1909, **62**, 226.)

Hair

Hair is one of the most stable of the keratoid substances, resisting the action of reagents to a marked extent. It is remarkable for the high proportion of sulphur contained in it,[2] and for the presence

[1] "Keratin" has been employed for coating pills intended to act on the small intestine. Its resistance to the gastric juice and solubility in the pancreatic juice render it very suitable for this purpose (see Bourqueolt, *Pharm. J.*, [3], 19, 1035).

[2] Marriott (*J. Soc. Leather Trades Chem.*, 1925, 9, 612) found 4.5% of sulphur in degreased human hair.

of a large proportion of silica in the ash left on ignition. The *ash* of hair ranges from 0.5 to 2.0%, and consists chiefly of sulphates of alkali metals, ferric oxide, and silica, the last constituent sometimes forming 40% of the total ash.[1] Vauquelin states that the iron and manganese present in dark hair are replaced in fair hair by magnesia, but this result requires confirmation.

Hair consists of a cylindrical keratinous tube covered with minute scales, the points of which are directed toward the free extremity. Hence three morphological elements are distinguishable: the cuticle, the cortex, and the medullary substance.

Hair is closely allied in structure and chemical composition to fur and wool, and no clearly defined distinction can be drawn between them. The fine hair on some animals closely resembles wool, whilst the coarse wool on others simulates hair. Wool is peculiar in the wavy or curling nature of the fibre, and is distinguished by the comparatively loose attachment of the outer scales. In hair, these scales usually lie so close to the stem that the serrations are scarcely perceptible, whereas in wool they are strongly in evidence (see Figs. 60 and 63). The structure of wool and hair has been fully described by Bowman (*J. Soc. Dyers and Col.*, 1885, **1**, 109 *et seq.*).[2]

The illustrations on p. 263 (taken from Swaine Taylor's *Medical Jurisprudence*) show the microscopic appearance of hair and fur from different sources. The magnifying power is 300 diameters in each case, except in that of No. 4, which is shown only 70 times the natural size.

The cells and the linear markings on the cortical portion afford the chief distinctions between hairs from different sources.

The hair of the human head appears under the microscope as transparent cylinders of various colours, and exhibits markings resembling (but less distinct than) those of wool. As a rule, the hair of women is finer and longer than that of men, and the hair of children finer and more silky than that of an adult. No. 2*a*, representing a transverse section of human hair, shows the cortical and medullary portions, and

[1] Gorup-Besanez (*Ann. Chem. Pharm.*, **66**, 321) found the hair of the lower animals to contain from 0.12 to 0.57 % of silica, whereas human hair contained only from 0.11 to 0.22 %.

[2] "The difference between wool and hair is rather one of degree than kind, and all wool-bearing animals have the tendency, when their cultivation is neglected, to produce hair rather than wool. Wool and hair, fur being intermediate, are simply modifications of the same root-substance, and the scales of the wool-fibre have a much larger free margin than is the case with hair, being only attached to the stem by about one-third of their length, and in many cases the ends are more or less turned outward, so as to present a much more serrated edge than is the case with hair. The interior of the fibre portion, however, does not differ in the least from that of hair, and can neither be distinguished from it chemically nor microscopically."—Bowman.

the air-cells in the interior of the cylinder. No. 3 represents the pointed extremity of a hair from the eye-brow. These hairs (and those from the eye-lashes) are thicker and coarser than hairs from the head, and are opaque, except toward the point. No. 4 represents the sheath of the hair (magnified 70 diameters) with the hair issuing from it, a condition which occurs when the hair has been pulled out violently from the skin. When a hair has been indented,

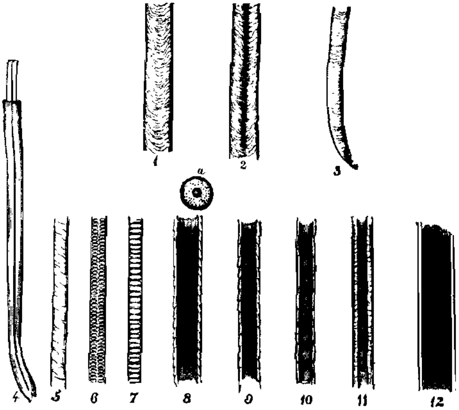


FIG. 15.—Microscopic characters of hairs.

1, Hair of child; 2, hair of adult (*a*, transverse section); 3, conical hair from eye-brow; 4, human hair (× 70) with tubular sheath, as torn out by force; 5, hair of spaniel; 6, fur of rabbit; 7, fur of hare; 8, horse-hair; 9, goat's hair; 10, hair of fox; 11, cow's hair; 12, hair of fallow deer.

cut, or bruised, the microscopic appearance of the medullary structure often shows marks of such treatment.[1]

The hair of the lower animals is coarser, shorter, thicker, and less transparent than human hair; but the hairs of the spaniel and skye-terrier are long, silky, and very similar to human hair. The diameter of the hair from a particular animal varies greatly, and cannot be regarded as more than roughly characteristic. The furs or hairs of the rabbit, hare, squirrel, mouse, rat, and other rodents are characterised by dark, transverse cells.

When colourless (white) hairs or feathers are boiled with diluted sulphuric acid, they dissolve to a colourless solution; but when black or brown, they yield a similarly coloured liquid, and a black or brown amorphous pigment remains undissolved. This pigment is insoluble in dilute acids or alkalies, and is with difficulty acted on by strong acids, except nitric acid. It is slowly acted on by bromine, forming a substance soluble in water, and is said to yield only traces of ammonia when ignited with soda-lime. The black pigment is present to the extent of about 1% in the feathers of the rook. A mean of 10 analyses of the black pigment from the feathers of several species of *Corvus* showed: Carbon, 55.4; hydrogen, 4.25; and nitrogen, 8.5% (Hodgkinson and Sorby, *J. Chem. Soc.*, 1877, 1, 427). According to Spiegler (*Beitr. Chem. Physiol. Path.*, (1903) **4**, 40 the pigment of hair contains sulphur.

The pigment of black hair and feathers is changed to yellow by treatment with hydrogen peroxide, an oxidising agent which is extensively employed for bleaching hair and feathers.[1]

Hair-dyes[2]

These may be divided into six classes,

(a) Substances of the type of henna, which react with keratin and can be considered substantive dyes.

(b) Metallic dyes, which make use of the labile sulphur in the hair, forming coloured sulphides.

(c) Vegetable dyes, such as walnut juice, etc., which develop their colour on exposure to air.

(d) Mixtures of metallic salts and a reducing agent, which form a coloured pigment, such as silver-pyrogallol dyes and permanganate.

(e) Coal-tar dyes, such as paraphenylenediamine, which require the use of hydrogen peroxide to develop the colour.

(f) Vegetable dyes, such as indigo leaves and chamomile, where the pigment is produced by the decomposition of a glucoside.

Many of these so-called hair-dyes are objectionable, and some are actually dangerous. Erysipelas and inflammatory swellings are liable to result, and well-authenticated cases of lead poisoning and paralysis have occurred from the use of preparations containing lead.

[1] To produce "golden hair," the grease is first thoroughly removed by washing the hair in soft soap and water, with the addition of a little ammonia. Hydrogen peroxide (of 20 volumes strength = 3% of available oxygen) is then applied to the hair.

[2] In compiling this section much use has been made of a book by Redgrove and Foan entitled "Blonde or Brunette" (Heinemann, 1929).

These dyes may be conveniently divided into three groups.

(a) Those which are perfectly harmless. These are mostly of vegetable origin, but exclude those which contain pyrogallol. Dyes which rely on the formation of iron sulphide with the sulphur of the hair or contain hydrogen peroxide or potassium permanganate are included in this class.

(b) Those which are slightly toxic. In this group are dyes containing pyrogallol and most of the metallic salts. Copper is possibly more dangerous than lead, while silver is only very slightly toxic. The concentration of these substances is, however, important.

(c) Those which are definitely dangerous. This group includes the so-called para dyes and allied coal-tar products. It also includes preparations containing mercury.

It is necessary to cleanse the hair very thoroughly before attempting to apply the dye, since a small trace of grease will produce very uneven results. The usual method is to employ a shampoo containing soap and little added alkali, such as sodium carbonate and borax, using water as the liquid medium. The so-called dry shampoos, which are applied as a liquid, are generally solutions of soap in a mixture of water and methylated spirit. On no account, however, should spirit containing pyridine be used, since pyridine is distinctly toxic.

A widely used article is composed of lead acetate, suspended sulphur, rose-water, and glycerin. In another, said to be capable of dyeing any shade from black to light brown, the free sulphur is replaced by sodium thiosulphate ("hyposulphite"). A chestnut-brown dye contains cupric nitrate and pyrogallic acid, while a black *dye is composed of silver and copper nitrates, with ammonia.* A preparation which dyes hair almost instantaneously consists of a solution of ammonio-nitrate of silver. This is applied to the hair first, and is followed by a solution of pyrogallic acid.[1] A hair-dye

[1] The *Lancet* for January 13, 1877, stated that of 18 samples (three American and the rest English) of so-called hair-restorers, including all the best known, fourteen were found to contain suspended sulphur and lead in varying but always in very considerable quantity. Many were described as "perfectly harmless," "free from injurious substances," etc., and only one was plainly stated on the label to be poisonous if taken internally.

Two other samples contained sodium thiosulphate instead of free sulphur. The handbill which accompanied one of these warned purchasers against dangerous hair-restorers containing lead, as likely to lead to paralysis and insanity, and recommended that *all* such preparations should be tested for lead by potassium iodide. This reagent gives no precipitate of lead iodide in presence of excess of sodium thiosulphate, so that the lead which existed in considerable proportion in the preparation in question would escape detection by the test recommended.

Another sample contained a considerable quantity of lead in solution, but no sulphur or thiosulphate. The remaining preparation was that referred to in the text as containing silver ammonio-nitrate and pyrogallic acid in separate bottles.

consisting essentially of a solution of potassium bismuth citrate has been recommended by Hager, and one containing ammonio-citrate of bismuth, with the addition of sodium thiosulphate, by Naquet (*Year-book Pharm.*, 1873, page 360; 1883, page 326).

If the statement that such preparations as the above are "restorers" and not dyes were correct, they should restore hair which was formerly red to its original colour.

In the second class amino-phenols or similar substances are increasingly employed as hair-dyes. These bodies, especially the salts of *m*-phenylene-diamine and diamino-phenol, resemble pyrogallol in undergoing rapid oxidation when their solutions are exposed to the atmosphere, with the formation of coloured bodies which will dye or stain the hair. According to a patent by Lumière, the dye is prepared by dissolving the hydrochloride or other salt of diamino-phenol in dilute alcohol, and adding sodium sulphite to prevent too rapid oxidation. By varying the strength, various shades of dye are obtained. Unsubstituted 2,6-diaminophenol may also be used. The range of shades produced by means of the "para" dyes is illustrated in the following table. (The figures indicate grm. per litre.)

	Black	Chestnut	Red-blonde	Blonde
Para-phenylene-diamine	20	5	2	1
Para-diamine phenol	0	5	10	.5

It is usual to add a small quantity of ammonia (0.5 to 1% of 0.880 ammonia) and small amounts of resorsinol are sometimes added.

In the case of henna dyes, additions of either iron or copper and pyrogallol are made in order to obtain a complete range of colours. Henna alone gives an auburn shade and is usually sold only as a shampoo powder. A typical formula is:—

Henna powder, 10 parts; soap powder, 40 parts; anhydrous sodium carbonate, 25 parts; borax, 15 parts; potassium carbonate, 5 parts; saponin, 4 parts; and perfume 1 part.

When used as a dye, henna preparations are applied as a paste. Typical formulae are given on the following page.

	Light brown	Brown	Dark brown	Black
Henna	100	100	100	100
Pyrogallol	5	5	10	15
Copper sulphate	5	7	8	10
Sienna	5	5	10	10
Lamp black	0	1	5	20

When henna preparations contain metallic salts and pyrogallol they are generally designated "henna-rastick."

Cases have been known where the use of such mixtures has produced peculiar shades, such as green. These abnormalities can generally be traced to insufficient mixing of the constituents (Redgrove and Foan, *loc. cit.*).

m-Aminodimethyl-*o*-toluidine or monomethyl-2:4-diaminoanisole have been recommended for dyeing hair, etc., (*Ger. Pat.* 230630). Also *m*-diaminoanisol or *m*-diaminophenetol (*Eng. Pat.*, 5134, 1910). *p*-Phenylenediamine is said to have an irritant effect on the human skin which may be counteracted by the addition of sodium sulphite in equal weight. The colour is subsequently developed with hydrogen peroxide, or these substances may be present in the same solution.

Colman (*Chem. Ztg.*, 1911, **35**, 899) recommends the use of 2.5 parts of toluylenediamine and 5 parts crystalline sodium sulphate in 100 parts of water. Hydrogen peroxide is added and the mixture applied to the hair.

Erlenbach (*U. S. A. Pat.* 992947) obtains blue black tints by using instead of certain mixtures of *p*-phenylenediamine and *m*-diamines, *m*-phenylenediamine, *m*-diaminoanisole, or *m*-diaminophenetole.

The Examination of Furs

Microscopical Examination.—An examination of the nature of a fur may be required if there is reason to suspect that a cheaper substitute has been supplied in place of a more expensive kind. The most common fur is that of the rabbit, which is often found masquerading under the scriptural name of "coney," and fortunately the characteristic structure of the hair of the rabbit (or hare) is not readily mistaken for that of animals of other genera. Illustrations of a few types of fur hairs will be found in Mitchell and Prideaux's "*Fibres Used in Textile and Allied Industries*," and in an article

by the same authors in *Knowledge* (1910, **36**, 283). These have been supplemented by a detailed account by H. E. Cox (*Analyst*, 1929, **54**, 694) of the microscopical characteristics of the long fully developed hairs of the principal fur animals. It should be noted that in most furs short hairs of the woolly type are also present, and that these show little signs of a medulla, and are not readily distinguishable in the fur of different animals.

The following particulars and photomicrographs are given here by permission of Dr. Cox:—

Rabbit.—These are mainly small fibres, about 2 cm. in length and 30–40μ diameter. Some are as wide as 80μ, and show 3, 4, 5, or 6 rows of medullary cells with well-defined longitudinal lines; the medullary cells in the larger hairs occupy almost the whole thickness. (Fig. 16.)

Hare.—These are similar to the rabbit hairs, but are longer and thicker. The hair may be 3 cm. or more in length and 100μ diameter, but show the characteristic medulla.

Cat.—These hairs are generally smaller than those of the rabbit; length about 2.5 cm. and width 15–35μ. The medullary cells are rectangular and some distance apart; they do not occupy the whole width as in the rabbit. The edge shows scales distinctly. (Fig. 17.)

Peschianiki (Russian Cat).—This fur differs materially from that of the ordinary cat. The fibers, though short and soft, are much thicker (about 100μ in the largest), and the medulla occupies most of the width, and is composed of thin-walled cells compressed in concertina fashion; the appearance is not unlike the scales of a fish in the wider hairs. (Fig. 18.)

Opossum.—Large thick hairs, 3–5 cm. long and 50–80μ wide. They are fairly uniform, and have a thick medullary layer occupying about half the width. The medullary cells are thick and distinct in outline. (Fig. 19.)

Skunk.—This shows hairs wider than the opossum, and with a very wide, ill-defined medulla, not showing definite cell walls; length 5 or 6 cm. The smaller hairs are much scaled, and generally there is more woolly hair at the base of the fur than with the opossum (Fig. 20.)

Nutria (Coypou or Otter).—Short woolly fibres about 2 cm. long and 20–30μ diameter, showing large scales but no medulla; fairly uniform. (Fig. 21.)

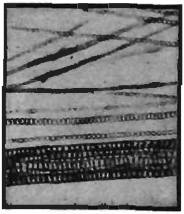


FIG. 16.—Rabbit.

FIG. 17.—Cat.

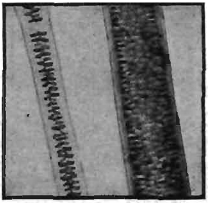


FIG. 18.—Russian Cat.

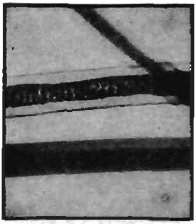


FIG. 19.—Opposum.

Fig. 20.—Skunk.

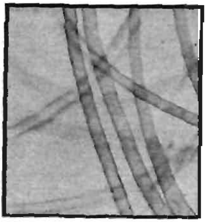


Fig. 21.—Coypou or Otter.

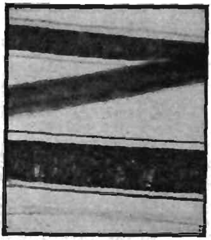


Fig. 22.—Sable.

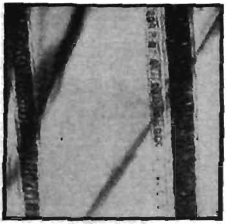


Fig. 23.—Fox.

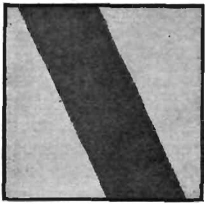


FIG. 24.—Goat.

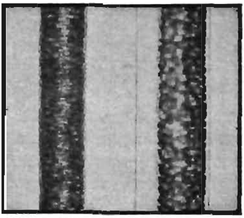


FIG. 25.—Wallaby

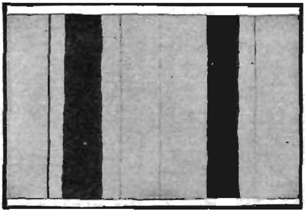


FIG. 26.—Racoon.

Sable (Weasel).—These are rather delicate hairs, though large. The width is up to 80μ, with a wide dense medulla interlocked with cells showing scale-like markings. The small hairs show barbed scales at the edge. (Fig. 22.)

Fox.—The fur has long hairs (6 or 8 cm.) with bearded edges; the width is about 50μ, and there is a dense thick-walled medulla occupying 80 % of the width. (Fig. 23.)

Goat.—The hairs are long and coarse and very dense; length up to 6 cm., and width 120 to 130μ. The medulla is thick, occupying 75 % of the width, and has a fish scale-like structure. (Fig. 24.)

Wallaby.—The hairs are about 3 cm. in length and 80μ in diameter; well defined, dense medulla occupying nearly the whole width, irregularly arranged cells not overlapping, as do those of the weasel; smooth edges (Fig. 25).

Racoon.—The fur has long coarse hairs, 5 or 6 cm. by 120μ; very dense medulla, 50μ wide, with smooth edge and indefinite structure; the sheath of the hair is smooth and transparent, showing no scales (Fig. 26).

Chemical Examination.—During the last few years the chemical examination of fur has become a very important matter, owing to the numerous cases of dermatitis which have been produced by irritation of the skin caused by the wearing of furs.

The question has been fully dealt with in two medical papers—one by Professor White (*Trans. Lond. Dermat. Soc.*, 1923, p. 41.) and the other in a Report on Fur Dermatitis to the Minister of Health (No. 27, 1924).

The view now generally accepted is that these cases must be attributed to active chemical agents left in or on the fur after the dyeing process, owing to incomplete oxidation during the formation of the dye and imperfect removal of the oxidising substances or intermediate products.

In the dyeing process the furs are either immersed in a bath containing the intermediate substance and the pigment subsequently developed by an oxidising agent, such as potassium chlorate, hydrogen peroxide, potassium chromate or ammonium vanadate; or the dye intermediate may be applied to the ends of the fibres by means of a brush and then developed.

More recently the finished dyes have been used for this purpose, and these are less likely to cause any irritation than the pigments formed by development on the fibres themselves.

Common Irritant Dyestuffs.—Cox (*loc. cit.*) gives the following list of the more common dyestuffs, which may possibly have an irritant action on the skin:—

(1) Meta-phenylene diamine; (2) para-phenylene-diamine; (3) toluylene-diamine 1:2:4; (4) toluylene-diamine 1:3:4; (5)[1] toluidine *o-*, *m- and p-*: (6) pyrogallol; (7) quinone; (8) hydroquinone; (9) para-aminophenol (ursol P.); (10) di-aminophenol (amidol); (11) para-methyl-aminophenol (methol); (12) di-amino-diphenylamine. The list is not exhaustive, but it is readily demonstrable by physiological experiment that all, except No. 5, are definitely irritant to the skin. Nos. 1, 2, 3 and 6 are the most common, and, of these, para-phenylene-diamine is perhaps the most frequently met with, and the most toxic. With regard to toluidine, Cox does not think it likely that it is irritant in small quantity to the normal skin, and certainly, when applied to his own arm, it did not produce any inflammation in 24 hours, although para-phenylene-diamine did so in an hour or two, and diamino-phenol raised blisters which persisted for days. It sometimes happens that two of these substances may be present in the same fur, as in the case of one specimen showing stripes of brown and black, due to pyrogallol and *m*-phenylene, respectively. Methoxy and ethoxy groups may be associated with para-phenylene-diamine, and naphthalene-diamines are a possibility.

All these substances are strong reducing agents and reduce silver nitrate; several are known as photographic developers, some of which cause irritation and blisters on the fingers of persons with sensitive skins. They also reduce Fehling's solution, with the production of various colours, but these colours are not sufficiently constant for diagnostic purposes, being much influenced by concentration and by other substances which may be extracted from the fur.

Identification Reactions.—The following reactions all apply to aqueous solutions in concentration 1 in 10,000; they are distinctly visible at this concentration, and many of them at 1 in 100,000 some even at greater dilutions, especially if viewed in column in Nessler glasses. They are sufficient for the identification of the substances named, though the colours may not be absolutely

[1] Toluidine is no longer used in dyeing furs (Private communication from Dr. H. E. Cox).

specific in all cases. It is necessary to confirm the reactions and not take one test as conclusive; thus the yellow colour with sodium nitrite is given by several other substances besides the commonest one—meta-phenylene-diamine; and, of course, there may be a mixture of two or more substances which will complicate the reactions. Care must be taken not to add too much reagent, especially when testing very dilute solutions. In the case of test (4) it is important that the solution should not contain any free acid, or chlorine may be liberated and a different reaction obtained; the solution should be neutral or very slightly alkaline, and a large excess of hypochlorite must be avoided, or the colour may be bleached. This test is not equally valuable in all cases; for example, the difference between the brownish-pink given by meta-phenylene-diamine or 1:2:4 toluylene diamine and the violet-blue of para-phenylene-diamine and 1:3:4 meta-toluylene diamine is distinctive, but, though the three toluidines give different colours when pure, it is not practicable to distinguish them in very dilute solution as extracted from fur.

In test (5) the solution should be distinctly acid with hydrochloric acid; this reagent, which is the familiar indole reagent of the bacteriologist, serves to distinguish *p*-phenylene-diamine from meta-toluene-diamine. In No. 6 about 2 drops of ferric chloride solution should be added to the neutral or faintly acid liquor, then hydrogen sulphide water 1 c.c. at a time; a large excess may mask the colour or liberate sulphur.

The reduction of Fehling's solution in the cold refers to the effect observed on adding 3 or 4 drops of mixed Fehling solution to 5 c.c. of the solution to be tested, not to the reverse addition of a little of the solution to be tested to a relatively large volume of the Fehling solution. It is generally advisable to use boiled cooled distilled water to eliminate oxidation as far as possible, and the solutions should be fairly fresh.

Oxidation Products.—The chemistry of the oxidation of these substances is obscure, and the composition of the final products appears to be unknown. Paraphenylene-diamine is of particular interest in this connection, because of the intermediate compounds formed during its oxidation and their possible toxicity. It seems certain that the first stage (according to Prof. Perkin) is the forma-

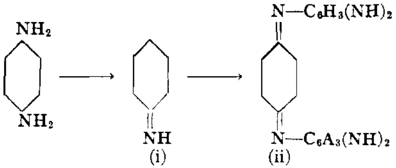
	Metaphenylene-diamine	Paraphenylene-diamine	1:2:4-Meta toluylene-diamine	1:3:4-Meta toluylene diamine	Para amino-phenol	Para-amino-diphenylamine
1. Dilute sodium nitrite added to cold acidified solution.........	Intense brownish yellow	Slight* brown colour (fades)	Orange colour	Brownish pink	Slightly yellow	Red
2. Excess of alkaline solution of -naphthol added to (1)........	Reddish brown colour	Brownish colour	Intense red	Dirty yellow or brown	Fluorescent green, afterwards turning brown	Yellow or brown
3. Bromine water...............	Slight white ppt.	Nil	Nil	Very faint ppt.	Nil	Brownish ppt.
4. Phenol (5 per cent solution) and 2 drops of sodium hypochlorite *added to neutral* solution.	Pink	Violet blue	Pink	Violet	Blue	Yellow
5. Alcoholic solution of *p*-dimethyl-amino-benzaldehyde added to acidified solution.	Bright yellow	Red	Yellow	Yellow	Yellow	Faint yellow
6. Ferric chloride.	Nil	Violet	Nil	Violet	Nil	Reddish violet
7. Hydrogen sulphide water added to (6)	Nil	Intense violet	Nil	Claret colour	Violet	Brick-red colour
		The dimethyl compound gives similar reactions but a blue colour with $FeCl_3$ and H_2S. Both give a pink colour with potassium cyanide	Reduces Fehling's solution in the cold			Violet colour with quinone

* As this cannot be a real diazo compound, it is probably an oxidation reaction.

	o-m-p-Toluidine	2:4 Diamino-phenol	Methyl-*p*-amino-phenol	Quinone	Hydroquinone	Pyrogallol
1. Dilute sodium nitrite added to acidified solution.	Nil	Bright red	Faint yellow	Nil	Faint yellow	Nil
2. Excess of alkaline naphthol added to (1)................	Bright red colour or ppt.	Brown colour	Slightly brown	Slight reddish colour	Red colour	Nil
3. Bromine water...............	White ppt.	White ppt.	Nil	Nil	Nil	Nil
4. Phenol (5 per cent solution) and 2 drops of sodium hypochlorite added to neutral solution....................	Brown at first, *o*- and *m*- subsequently become blue	Red colour	Blue-violet develops slowly	Nil	Nil	Brownish
5. Alcoholic solution of *p*-dimethyl-amino-benzaldehyde (acidified).	Greenish yellow	Yellow	Nil	Slight yellow	Slight yellow	Slight yellow
6. *Ferric* chloride..............	Nil	Nil	Nil but turns green on *adding* H_2S	Nil	Nil	Reddish turning greenish-black
7. *Mercuric acetate* solution	Nil	Purple colour	No colour (very slight pink on standing)	Pale yellow with *mercuric* sulphate	Nil	Yellowish
8. Potassium *cyanide* solution....	Nil	Greenish blue	Yellow slowly develops	Brownish	Yellow	Pink
				Reduces Fehling's *solution* cold. Gives purple-*black colour* with ammonical copper *chloride*; also violet colour *with p*-amino-diphenylamine		Gallic acid gives bright red with mercuric acetate

Cox finds that a more sensitive test for *p*-phenylene-diamine than those given in the foregoing table is to add to the solution a fewdrops of aniline hydrochloride solution, followed by a drop of a dilute solution of potassium *dichromate or permanganate.* This gives a green indamine, and on boiling, the *colour changes* to pink owing to the formation of a safranine. This reaction is even more sensitive than the formation of Lauth's violet.

tion of quinone di-imide (i), and the second Bandrowski's base (ii) thus:



Some controversy centres round this substance. Gordon (*Trans. Med.-Legal Soc.*, 1926, **20**, 73) shows that it is non-irritant and expresses the opinion that it is the proper end-product in fur-dyeing. This seems to be open to question, as, although the constitution of finished black or brown (in its many forms) is not settled, it is clear that it has at least 8 rings, and that Bandrowski's base is not the final product, nor is it a fast colour. Gordon attributes the irritant properties of fur dyed with para-phenylene-diamine to a substance which he calls "A," the constitution of which is not stated, but which gives a coloured solution with water, whereas the base B. is insoluble; it would appear that A. is the di-imide. He identifies A. by spectroscopic comparison. In Cox's observation of these dyes and their intermediate products there is only a general absorption visible spectroscopically, not a specific one, so that one cannot differentiate with certainty the colour due to A. from that of other, possibly similar, bodies, or even some finished dyes. It may be further remarked that Bandrowski's base is easily reduced to the form of a leuco compound (*Ber.*, 1894, **27**, 482), so that it may, in appropriate circumstances, be an indirect cause of irritation. In Cox's experience, Bandrowski's base is always associated with some partially oxidised para-phenylene-diamine, which can be detected by the reactions given.

Extraction of the Fur for the Tests.—For the application of these tests, extracts from the fur must be prepared; this may be done by cutting the fur from the skin as closely as possible (sometimes it is desirable to soak the skin also), de-fatting with petroleum spirit, then extracting with (1) cooled boiled water, (2) dilute (1%) acetic acid for at least 24 hours. All the solutions should be preserved from atmospheric oxidation as much as possible. The petroleum

spirit should be shaken with acidified water to extract from it any bases. The object of using weak acetic acid is to simulate the action of perspiration which in contact with the fur may be an agency in setting up the irritation. Some furs which are easily wetted may be soaked without de-fatting.

The extract so prepared should be tested for inorganic substances and for acids, as well as for dyestuffs or intermediates. Well-dyed furs yield no colour when so treated, other than a trace of dirt; those containing diamines are usually of a distinct brown or pink colour, which is an unfavourable sign. Decolorisation of the extract with charcoal is inadvisable, as it may remove traces of the important substances.

It is interesting to make an approximate estimated of the amount of any substance detected in the extract; this can be done colorimetrically by comparison with known solutions containing the same volumes of reagents.

Wool

Wool is closely allied in composition and general characteristics to fur and hair (page 261), but is usually more elastic, flexible, and curly; and the flattened scales, which are observed under the microscope to cover its surface (Fig. 6), give it the property of matting with greater readiness than is the case with either fur or hair. This difference is less apparent in raw wool than in that which has been scoured and treated with a dilute acid.

The microscopic characters of wool have been described and illustrated at length by Bowman (*J. Soc. Dyers and Col.*, 1885, **1**, 86) and Watson Smith (*ibid.*, 1889, **5**, 12).[1] The application of X-ray analysis in revealing the characteristic fine structure of different wools is dealt with by Ewles and Speakman, *Proc. Roy. Soc.*, 1930, **105**, B, 600.

The length of the wool fibre varies between 1.5 and 7 in. and its diameter from 0.004 to 0.0018 in. Bowman gives the following data:

[1] Sheep's wool varies enormously in the diameter of the fibres and in other characteristics. The chief varieties of wool from animals other than the sheep are: *Cashmere wool*, from the fine downy hair of the Cashmere goats. *Vicuna wool*, now little used, from the very slightly curly hair of the llama or vicuna goat. *Alpaca wool*, similar to, but not quite so fine as, vicuna wool, from the alpaco or pako goat of Peru. *Mohair*, or so-called "camel's wool," from the long, slightly curly, silky hair of the angora goat.

A mixture of ordinary sheep's wool with the hair of hares or rabbits is sometimes substituted for vicuna wool. The term "viguna" or vicuna wool is now applied in the wool trade to a mixed fabric of silk and cotton.

Nature of wool	Breaking strain in grm.	Elasticity in % elongation	Diameter of fibre in inches
Leicester	502	0.284	0.00081
Southdown	86	0.268	0.00099
Amstration Merino	50	0.335	0.000517
Saxony	39	0.272	0.000338
Mohair	586	0.299	0.000170
Alpaca	149	0 242	0.000526

Raw wool is far from being an approximately pure keratoid substance. Besides moisture and accidental dirt it contains a considerable proportion of *suint* or wool-soap (page 281), sometimes called "yolk."

FIG. 27.—Sheep's wool (highly magnified), showing the cortical scales and the medullary tube.

The estimation of moisture in wool is an important point. Bowman returns the natural moisture (at 38°) as 8.28% on the average, with the limits 7–10% and as 14% (at 100°). At the English Testing Houses the following percentages of moisture are allowed: Worsted yarn, 18.25%; carded woollen yarn, 17%; noils, 14%; scoured wool, 16% (the silk standard is 11%).

Hummel (*The Dyeing of Textile Fabrics*) has suggested the following method for the analysis of raw wool:

Moisture is determined by drying the wool at 100° in a current of hydrogen or other inert gas.

Wool-fat is determined by extracting the sample with ether. The solution also contains more or less of the oleates of the wool, which are separated from the wool-fat by agitating the etheral solution with water.

The residual wool is washed repeatedly with cold distilled water, by which more *oleates* are extracted. The solution is added to the water separated from the ether. The wool is next washed with alcohol, and the oleates dissolved added to those recovered from the aqueous solution. The wool is then treated with dilute hydrochloric acid, which removes earthy oleates not soluble in water.[1] The wool is washed with cold water to remove acid, dried, and treated in succession with ether and alcohol. On evaporating these solvents to dryness, a residue of oleic acid is obtained, from which the amount of pre-existing *earthy oleates* can be calculated.

The wool, freed in the foregoing manner from fatty and soapy matters, is dried and teased out over paper to remove dirt, sand, etc. The purified *wool-fibre* is then carefully washed on a fine sieve, dried at 100° and weighed, the amount of *sand* and *dirt* being estimated by difference.

The following analyses, by Märcker and Schulze, were made by the foregoing method, and illustrate the results yielded by typical raw wool.

	Wool of lowland sheep	Wool of full-bred rambouillet sheep	"Pitchy" wool
Moisture	23.48	12.28	13.28
Wool-fat extracted by ether	7.17	14.66	34.19
Wool-soap extracted by subsequent treatment:			
By water (wool-sweat)	21.13	21.83	9.76
By subsequent treatment with alcohol	0.35	0.55	0.89
By subsequent treatment with dilute hydrochloric acid	1.45	5.64	1.39
By subsequent treatment with ether and alcohol	0.29	0.57	....
Pure wool-fibre	43.20	20.83	32.11
Dirt (by difference)	2.93	23.64	8.38
	100.00	100.00	100.00

[1] In the case of very dirty wool a considerable quantity of lime is dissolved which has its origin in calcareous dust and not in lime-soap.

The following analyses by Faist further illustrate the general composition of sheep's wool (air-dried).

Description	Mineral matter	Suint and fatty matter	Pure wool	Moisture	Pure air-dry wool
Raw Wools:					
Hohenheim, with little suint	6.3	44.3	38.0	11.4	49.4
Hohenheim, with much glutinous suint	16.8	44.7	28.5	7.0	35.5
Washed Wools:					
Hohenheim	0.94	21.00	72.00	6.06	78.06
Hohenheim, with difficultly soluble suint	1.3	40.0	56.0	2.7	58.7
Hungarian, very soft	1.0	27.0	64.8	7.2	72.0
Würtemburg, less soft	1.2	16.6	77.7	3.5	82.2

Wool-fat (Vol. II, page 741) is remarkable for containing a large proportion of *cholesterol* and *isocholesterol*. It takes up a considerable proportion of water. Purified wool-fat forms the lanolin of pharmacy.

Suint, Wool-sweat or Wool-soap, is the portion of the wool-soap soluble in water. It has a very complex composition, and has been the subject of numerous researches. Suint consists chiefly of the potassium salts of the higher fatty acids, including cerotic acid; with smaller quantities of valerate, butyrate, propionate and acetate; and phosphates, sulphates, and chlorides. Ammonium compounds are present in small proportion.

The *mineral matter* of purified sheep's wool ranges from 1 to 2%, and consists largely of sulphates of the alkali metals. It is remarkable for containing a notable proportion of silica.[1]

Cholesterol is contained in the wool suint (or yoke) which is removed in the process of wool washing. It melts at 145° (isocholesterol melts at 132°). It has a neutral action, is insoluble in water, soluble in boiling alcohol, ether, chloroform, sublimes at 199°. The Chemistry of cholesterol is dealt with by McLean and McLean (*Lecithin and Allied Substances*, monographs on Biochemistry, Longmans, Green & Co., (1927).

Wool-gelatin, according to Gardner and Carter (*J. Soc. Dyers, & Col.*, 1898, **14**, 167), is present in small proportion (1.65%).

[1] A sample of Lincoln wool which had previously been scoured by soap, thoroughly washed with pure water and dried, was found by Wood (*J. Soc. Dyers and Col.*, 1885, 139) to yield 1% of *ash* of the following composition:

	Percentage composition		
	Total ash	Ash soluble in water	Ash insoluble in water
K_2O	31.1	42.3	trace
Na_2O	8.2	17.3	trace
CaO	16.9	4.5	51.2
Al_2O_3 and Fe_2O_3	12.3	3.6	37.7
SiO_2	5.8	4.1	11.1
SO_3	20.5	24.8	trace
CO_2	4.2	3.4	......
P_2O_5	trace	trace	trace
Cl	trace	trace	......

Bowman found 2% of magnesia in the wool-ash from sheep which had pastured on a magnesian-limestone district.

Wool-keratin, according to Bowman (*J. Soc. Dyers* and *Col.*, 1885, **1**, 136), has the following composition:

	Carbon	Hydrogen	Nitrogen	Oxygen	Sulphur	Loss
Lincoln wool	52.0	6.9	18.1	20.3	2.5	0.2[1]
Irish wool	49.8	7.2	19.1	19.9	3.0	1.0[1]
Northumberland wool	50.8[1]	7.2	18.5	21.2	2.3	.....
Southdown wool	51.3[1]	6.9	17.8	20.2	3.8	.....
Mean	50.27	7.05	18.37	30.40	2.90	......

[1] The "loss" in the first two analyses represents the mineral matter. In the last two this is included in the carbon.

The mean of Bowman's analyses of wool-keratin correspond to the empirical formula: $C_{43}H_{71}O_{13}N_{13}S$.[1]

According to Rimington (*Biochem. J.*, 1929, **23**, 41) the sulphur content of wool varies with the breed of the animal. The following figures give an indication of the extent of the variation.

[1] Mills and Takamine (*J. Chem. Soc.*, 1883, **43**, 142) regard wool-keratin as a substance of definite composition, having the empirical formula $C_{43}H_{157}O_{14}N_{5}S$. This is based on the mean of the analyses of wool-keratin recorded in Gmelin's *Handbook of Chemistry* (**18**, 351); but Whiteley (*Proc. Chem. Soc.*, 1886, **49**, 142) pointed out that by a misprint the respective percentages of nitrogen and hydrogen were transposed. The corrected average composition of keratin, according to the average of the analyses in question, is C, 49.96; H, 7.11; N, 16.65; S, 3.39; and O (by difference), 22.89%. These figures correspond with the formula: $C_{41}H_{77}O_{14}N_{12}S$.

	Sulphur (on dry weight) %
Crossbred 50's (i)	3.46
Crossbred 50's (ii)	3.47
Crossbred 50's (iii)	3.54
Devon lamb	3.34
Welsh mountain	4.08
Cape merino	3.67
Turkey mohair (coarse)	3.02
Turkey mohair (fine)	3.18

When wool is heated to about 140° it begins to decompose, and on heating more strongly it yields an oily substance of intolerable odour, together with large quantities of pyrrole, much hydrogen sulphide, a small quantity of carbon disulphide, and mere traces of oily bases (*Ann. Chem. Pharm.*, **109**, 127).

Wool is affected to an appreciable extent by prolonged boiling with water, sensible traces of hydrogen sulphide being evolved. Bowman states that wool which looked quite bright when well washed with tepid water, became quite dull and lustreless under treatment with boiling water, and the appearance under the microscope was materially altered. Speakman (*Trans. Faraday Soc.*, 1929, **25**, 169) finds that water above 90° enables a permanent set to be imparted to the wool, and suggests that some form of internal re-arrangement occurs at high temperatures (see also Speakman, *J. Text. Inst.*, 1927, **18**, T 431; *Proc. Roy. Soc.*, 1928, *B*, **130**, 377). In presence of only a very small quantity of alkali, the prolonged action of boiling water gelatinises wool more or less completely.

Concentrated solutions of sodium hydroxide (38%) do not affect the elastic properties of wool, provided the liquor is only allowed to act for 5 minutes at 19° (Speakman, *J. Soc. Chem. Ind.*, 1929, **48**, 321 T).

Wool is not damaged to any great degree by solutions of soap, borax, ammonium carbonate, or carbonate of alkali metal, but is acted on by alkali hydroxides, even when very dilute. All alkalies, however, affect the disulphide grouping of keratins to some small extent (Marriott, *J. Soc. Leather Trades' Chem.*, 1928, **12**, 216). This action, however, is not visibly apparent, but it leads to a diminution in the sulphur content and an increase in the nitrogen content of the residual wool or hair. Knecht found that when wool was boiled for 3 hours with water containing sodium hydroxide in amount equal to 0.3% of the weight of the wool, the fibre was not disinte-

grated, but when the alkali was increased to 0.6% complete disintegration and almost complete solution of the wool took place.

Schützenberger obtained the following products by decomposing purified wool with an aqueous solution of baryta, under pressure, at 170°:

		%
Nitrogen evolved as ammonia		5.25
Carbon dioxide (separated as $BaCO_3$)		4.27
Oxalic acid (separated as BaC_2O_4)		5.72
Acetic acid (by distillation and titration)		3.20
Pyrrole and other volatile products		1 to 1.5
Percentage composition of fixed residue; containing leucine, tryosine, and other volatile products	C	47.85
	H	7.69
	N	12.63
	O	31.83

By distilling wool (flannel) with concentrated sodium hydroxide, *Williams* obtained a distillate containing a large quantity of ammonia, besides butylamine and amylamine. The ammonia is derived from the amide nitrogen, the cystine and the arginine grouping.

A fairly accurate separation of wool from cotton and linen can be effected by boiling the mixed fibres for some time with a solution of sodium hydroxide of 1.05 sp. gr. This dissolves the wool, leaving the vegetable fibres unaffected.

On the contrary, the vegetable fibres are affected by acids far more readily than wool, which fact is extensively employed for the recovery of the wool from waste fabrics of mixed nature.[1]

The determination of small quantities of wool in cotton materials may be carried out as indicated by Heerman (*Chem. Ztg.*, 1913, 1257). Sulphuric acid (80%) dissolves cotton completely in 2–3 hours, whilst wool only loses 1.5% of its weight in the same time. In practice the sample (5–10 grm.) is thoroughly extracted with ether and then with 96% alcohol, and the fibre is then treated with ten to twenty times its weight of the acid. After standing for 6 hours the cotton has completely dissolved. The liquid is diluted with water (cold) and any wool present remains undissolved; it may be dried at 105° after a thorough washing with water and weighed (an allowance of 17% being made for moisture).

By dissolving carefully washed wool in concentrated baryta water, passing carbon dioxide, and treating the filtered liquid with lead

[1] The goods are exposed in a closed chamber to the action of hydrochloric acid gas for a number of hours. When taken out and passed through suitable machinery the cotton falls to a fine dust, while the wool-fibre or shoddy remains practically unchanged. A similar method (in which sulphuric acid is commonly preferred) is employed for freeing raw wool from the "burrs" frequently observed in it.

acetate, a precipitate is obtained which, when washed, suspended in water, and decomposed by hydrogen sulphide, gives a solution which, after filtration and evaporation to dryness, yields a dirty yellow, non-deliquescent, acid substance. This body Knecht (*J. Soc. Dyers & Col.*, 1889, **5**, 72) called lanuginic acid. It dissolves slowly in cold, but readily in hot water, is sparingly soluble in alcohol, and insoluble in ether. The aqueous solution of lanuginic acid is not coagulated by boiling, but it precipitates both acid and basic colouring matters, forming lakes, and it also yields precipitates with tannin and with potassium bichromate. The compounds of lanuginic acid with colouring matters appear to be definite in composition, and precipitation by night-blue or picric acid may be conveniently employed for their determination. With phosphotungstic acid and with Millon's reagent lanuginic acid behaves like the proteins.

When heated to 100°, this substance becomes soft and plastic, and this property is shared by its lakes, most of which actually melt at the temperature of boiling water. This so-called acid becomes anhydrous at 110°, and on ignition leaves a considerable quantity of ash, consisting chiefly of barium carbonate. By substituting sodium carbonate for baryta in the preparation of lanuginic acid, Knecht obtained a product identical in external properties and all other characteristics with that previously prepared, but leaving a much smaller quantity of ash on ignition. Knecht found this substance to have the following ultimate composition: C, 41.61; H, 7.31; N, 16.26; S, 3.35 and O, 31.44%.

By heating wool with five times its weight of water to a temperature of 200–230° for 4 hours, the substance almost wholly dissolved, with liberation of hydrogen sulphide, etc., and the solution contained the lanuginic acid. Very similar products were obtained by heating horn and human epidermis with water to 200° under pressure.

When wool is boiled with a solution of sodium hydroxide to which a little lead acetate has been added, it dissolves, with brown or black coloration, due to the formation of lead sulphide. It appears, however, that the whole of the sulphur of wool does not enter into this reaction, a portion being in a more stable state of combination. Thus Chevreul found that by treating wool with alkalies the greater part of the sulphur is removed, but he was unable to extract the whole of it in this manner. By steeping wool 28 times in lime-water for 24 hours each time, and washing with hydrochloric acid between

each treatment, he succeeded in reducing the proportion of sulphur to 0.46%. The wool treated in this manner was no longer blackened by boiling with lead acetate and excess of alkali hydroxide. Knecht found that lanuginic acid also gives a negative reaction with the alkaline lead reagent, from which fact he considered it probable that the residual sulphur of the wool exists as lanuginic acid, or as some substance which readily yields lanuginic acid as a first product of its decomposition by reagents.

The blackening of wool by boiling with an alkaline solution of lead affords a ready means of distinguishing it from silk, cotton, and linen, neither of which fibres contains sulphur. The recognition of wool in mixed fibres may also be readily effected by the microscope (see further, page 278).

Knecht states that the plumbate reaction is also given with "stoved" silk (sulphur bleached) but this seems doubtful. *p*-Benzoquinone (Scharwin, *Z. angew. Chem.*, 1913, 254) gives with wool (and silk?) a rose tint darkening through red to almost black.

Prevention of Moth.—Several patented methods to treating wool are now in vogue for preventing the depredations of the larvae of the common clothes moth. The substances used are diazoaminobenzene, triphenylguanidine, phthalic acid, etc. The wool is impregnated with small quantities of these chemicals, and the treatment is much superior to the older method of using naphthalene.

In order to detect damage in the wool fibres, Sieber (*Textilber.*, 1928, **9**, 326) recommends staining with a solution of benzopurpurin 10 B. Undamaged fibres are not coloured, mechanically damaged fibres are stained pink, alkali damage gives yellowish pink to red shades, while acid damage shows as bluish-red to red. Sieber states that these colour changes are best seen in ultra-violet light. On the other hand, Burgess (*J. Text. Inst.*, 1928, **19**, T, 315) prefers to make use of the fact that tyrosine is absent in the epithelial scales and present in the cortical, and suggests the use of a mixture of sodium sulphoanilate and sodium nitrite for determining whether or not the wool has been damaged. Loss of the scales produces a pale yellow to deep red colour, the shade depending on the extent of the damage.

If moistened wool is treated with chlorine gas in excess, the chlorine is absorbed with great evolution of heat, hydrochloric acid is formed in large amount, and the wool is converted into a white pulpy substance. By using a limited quantity of chlorine diluted

with air, the wool remains intact, but becomes more transparent, acquires a glossy, silk-like appearance, a crackling feel, and an increased capacity for absorbing dyes. When chlorinated in the foregoing manner, wool does not readily acquire a yellow colour by subsequent treatment with soap or alkalies (*J. Soc. Dyers and Col.*, 1898, **14**, 175).

The chlorination of wool has been the subject of an English patent (1897, No. 11917).

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See page 251, also Glaister. *A Study of Hairs and Wools*, Cairo, 1931, and the works cited on pp. 260, 262, 264, 278, 281.

THE EXAMINATION OF FOODSTUFFS FOR VITAMINS

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FOREWORD

The manuscript of the following article was submitted early in 1931. Since then great advances have been made in the study of the vitamins and in the elaboration of methods of their estimation. Reference to the more important of these is made in footnotes and an appendix added in July 1932, but for a comprehensive review of the present position the reader is advised to consult the 3rd Edition of the monograph prepared by the Medical Research Council, "The Vitamins" Report No. 167. H. M. Stationery Office. London. 1932.

Within recent years the problem of the examination of foodstuffs for vitamins has become one of major practical importance. In many respects the methods at present in use are unsatisfactory, but the rate at which progress is being made in effecting improvements is such as to encourage the hope that reliable and generally approved techniques will soon be available, at any rate for the estimation of the more clearly defined members of this class of food accessory substance.

Another source of disturbance in biological tests, particularly those in which growth of the animal is taken as the criterion of the presence of the vitamin, is the confusion at present prevailing regarding the single or multiple character of some of the factors.

This is particularly well illustrated by the recent development in the study of what was formerly known as the "water-soluble B vitamin." At one time this factor was believed to be necessary for both the growth and the normal functioning of the animal organism, and it was held that the condition in man known as beri-beri and the somewhat similar condition in birds known as avian polyneuritis, resulted from a deficiency of this substance in the diet. No sooner had biological tests for this vitamin been established on what then appeared to be a satisfactory basis than the discovery was made that two distinct physiologically active factors were concerned, at any rate in the tests based on a restoration of growth.

Further work has now revealed that a positive response in the former "growth-test" on young rats for "water-soluble B" requires the presence in the supplement being administered of at least three, and possibly four, distinct substances of the vitamin class. It is, therefore, clearly unwise to define the character of a "growth-test" until there is reasonable certainty that its course will not be disturbed by deficiencies other than the one being studied.

There is, therefore, much to be said in favour of relating the biological methods of assay of vitamins, whenever possible, to specific disorders of the organisms rather than to the more general phenomenon of growth.

The accepted methods for the assay of vitamins C, B and D have this sound foundation, but, as yet, the character of the disorders associated with a diet deficient in the other members of the class is insufficiently recognised to serve as the basis of a practical test.

THE VITAMINS

The following is a very brief review of the more important facts concerning the vitamins. For more detailed information the reader is referred to one of the many text books and monographs dealing with the subject.[1]

Vitamin A.—The "vitamin A" of plant foodstuffs is probably identical in all cases with the pigment carotene $C_{40}H_{56}$.[2,3] The

[1] The Vitamins. Sherman and Smith. 2nd Ed. American Chemical Society Monograph. New York, 1931.
The Vitamines. C. Funk. Translation by H. E. Dubin of 2nd German Edition. Baltimore, 1922.
Medical Research Council Report No. 38. 2nd Edition 1924. 3rd Edition. No. 167, 1932.

[2] For a full description of carotene refer to "Carotinoids and related pigments." L. S. Palmer. Amer. Chem. Soc. Monograph. 1922.

[3] For a review of the recent work on the relation between carotene and vitamin A see Gulland. *J. Soc. Chem. Ind.*, 1930, **49**, 839.

formation and distribution of this pigment in plant tissues show a close parallel with those of the substance possessing the physiological action associated with "vitamin A."

The highly purified pigment will replace the vitamin in the diet of the animal. It is believed to undergo a transformation in the tissues of the animal, the nature of which is at present not known. The substance formed is the "vitamin A" that had been previously recognised as a component of certain fish liver oils and other animal foodstuffs. This substance is colourless, shows reactions recalling its relation to its lipochrome "provitamin" and an absorption band in the ultra violet spectrum with a maximum at $328\mu\mu$ (Morton and Heilbron, *Biochem. J.*, 1928, **22**, 987).[1] The function of carotene in the animal body is not known. Either the pigment itself or the product of its transformation in the tissues is essential for the growth of the young organism and plays an important part in maintaining the resistance of the tissues against bacterial infection. This has been demonstrated in a very remarkable manner by the recent observations of Mellanby and Green (*Brit. Med. J.*, 1929, **ii**, 691; 1929, **i**, 984; *Brit. J. Exp. Path.*, 1930, **11**, 81).

There is as yet no evidence that carotene is converted into or accompanies a substance resembling "vitamin A" in plant tissues. The problem of assaying the "vitamin A" value of plant foodstuffs is probably, therefore, that of estimating the pigment itself.

The foodstuffs of animal origin can be divided into two groups. In one are placed those which contain both carotene and "vitamin A," whilst in the second are those which contain the colourless "vitamin A" but in which no physiologically active pigment is present. Butter is an example of the former, whilst many fish liver oils are characteristic of the second type.

Vitamin B Complex.—The factor formerly known as "water-soluble vitamin B" is usually referred to now as the "vitamin B complex" in view of the discovery that it contains a number of individual substances essential for the animal organism. At the moment there is some confusion regarding the differentiation of these factors, but the table on p. 292 summarises the position to date (February, 1931).

With the exception of the factor B_1, comparatively little is as yet known about the formation of these substances. Green plant

[1] The work of Karrer, Morf and Schöpp (*Helv. Chim. Acta*, 1931, **14**, 1036) and of Heilbron, *Morton* and *Drummond* (*J. Soc. Chem. Ind.*, 1932) indicates that vitamin A is an unsaturated alcohol $C_{20}H_{30}O$ related to half the carotene molecule.

TABLE 1.—COMPONENTS OF VITAMIN B COMPLEX

Vitamin (British nomenclature)	American nomenclature	Alternative name or names	Sources	Effects produced in animals by diet deficient in this factor	Properties	Character
B_1	B — P[1] (beri-beri preventive) F vitamin.[2]	Anti beri-beri vitamin. Antineuritic vitamin. Torulin.[3]	Brewers' yeast, wheat germ, malt.	Inhibition of growth, degenerative changes in certain organs, atrophy of intestinal walls, inco-ordination and convulsions	Stable $pH < 7$ to 100° rapidly destroyed pH 7.	Probably a simple nitrogenous base.
B_2	P — P[1] (pellagra preventive) G vitamin.[2]	Antidermatitis vitamin.[4] Antipellagra vitamin. Possibly the vitamin D of Funk.[5]	Yeast, liver, egg white.	Failure to grow. Typical skin lesions possibly related to those of human pellagra. Loss of hair.	Stable to heat in acid solution. Opinion divided whether stable to hot alkalies.	Probably neutral or faintly acidic substance.
B_3	B_3[6]	....	Yeast.	Failure to maintain weight in pigeons.	Readily destroyed in warm acid solution, slowly destroyed at room temperature by alkalies.	Unknown.
B_4[7]	....	Possibly the "vitamine d'utilisation nutritif" (Lecoq)[8]	Yeast extract.	Loss of weight, muscular weakness and inco-ordination of movement distinct from that associated with B_1 deficiency.	Destroyed by alkalies, moderately stable to acids.	Unknown.
B_5	....	The factor Y of Chick and Copping.[9]	Yeast extract, egg yolk, liver.	Failure of growth.	Stable to alkalies.	Unknown.

[1] Goldberger, Wheeler, Lillie, and Rogers. *U. S. Pub. Health Rep.*, 1926, **41**, 297.
[2] Sherman and Axtmayer, *J. Biol. Chem.*, 1927, **75**, 207.
[3] Kinnersley and Peters. *Biochem. J.*, 1925, **19**, 820.
[4] Chick, Copping and Roscoe, *ibid.*, 1930, **24**, 1748.
[5] Funk and Lecoq. *Compt. rend. Soc. Biol.*, 1927, **97**, 440.
[6] Williams and Waterman. *J. Biol. Chem.*, 1928, **78**, 311. *N.B.* This is not the factor B_3 of Reader (*Biochem. J.*, 1929, **23**, 689) which is now referred to as B_4.
[7] Reader, (*Biochem. J.*, 1929, **23**, 689 described as B_3, now referred to as B_4. Reader (Biochem. J., 1930, **24**, 77). (cf. Williams and Eddy, *Carneg. Inst. Year Book*, 1928, **27**, 375).
[8] "*Recherches experimentaux sur les vitamines B.*" Thesis. Paris, 1928.
Peters. *J. State Med.*, 1930, **38**, No. 1, Jan. 20.
[9] Chick and Copping, *Biochem. J.*, 1930, **24**, 1764.

tissues contain relatively large amounts of B_1 and B_2, and they are also found in seeds, particularly in the embryos. Although yeast and yeast extracts have been used extensively for the preparation of concentrates of the various B factors, it appears probable that the vitamin value of the yeast is to a large extent determined by that of the medium in which cultivation is made. This is particularly true of the factor B_1, which, in the case of some yeasts, may be derived almost entirely from the medium. The information regarding the other factors is not so definite but it tends to support the view that yeast removes other vitamins than B_1 from the medium.

Vitamin C.—This substance, also known as the anti-scorbutic vitamin, appears from the work of Zilva[1] to be a single substance. Its chemical nature is unknown.[2] Under certain conditions, it can be precipitated from lemon juice preparations by lead acetate. It rapidly becomes inactivated in neutral and alkaline solutions, but it is relatively stable when stored at reactions about pH 1.0.

Zilva believes that the activity of the vitamin is controlled by the presence of a reducing principle which in itself is not active as an antiscorbutic. The vitamin is present in a large number of fresh fruits and vegetables and its formation during the germination of seeds has been demonstrated.

There is no information throwing light on the part played by this vitamin in the animal. In species susceptible to scurvy (man, monkeys, guinea-pigs) the appearance of the disease is quite definitely associated with the consumption of diets deficient in vitamin C and the disorder is rapidly cured by the administration of extracts supplying the factor. Certain species (*e.g.* the rat) are able to thrive on diets which produce scurvy in a susceptible animal. It is not known whether this indicates that vitamin C is not required for their metabolic cycle or whether their tissues possess the power of synthesising the molecule.

Vitamin D.—This factor plays an essential part in the complicated process of bone-formation in the growing animal. This part is not fully understood, but to some extent it appears to concern the interchange of calcium and phosphate between the contents of the gut and the circulating fluids of the body. The deposition of calcium

[1] In a recent paper Zilva (*Biochem. J.*, 1930, **24**, 1687) gives a general review of his investigations on the nature of the antiscorbutic vitamin C.

[2] Evidence has recently been obtained that vitamin C is a lexuronic acid. (Szent Gyorgi. *Nature*, 1932, **129**, 576, 690. King and Waugh. *Science*, 1932, **75**, 357.)

phosphate, or the complex carbonate-phosphate, in growing bone has been shown to follow simple physico-chemical laws.

Two mechanisms may be concerned in the deposition of lime salts in cartilage.[1] Super-saturation of the blood plasma with respect to bone salt would, of course, result in deposition, but although measurements of concentrations in normal plasma suggest that the solubility product is often exceeded, such figures are actually misleading owing to uncertainty as to the extent to which ionisation is depressed by the presence of plasma proteins and other organic substances.

The second mechanism concerns the phosphatase system present in the matrix which by hydrolysis of the phosphoric ester known to be in blood brings about a rise in the concentration of phosphate ion. This would, in the presence of a system saturated to bone salt, lead to deposition. Robison believes that both mechanisms play a part in the normal calcification of the skeleton. As far as is known vitamin D plays no part in this phase of calcification. It stabilises the calcium phosphate absorption and prevents the reexcretion that is often responsible for a negative balance. How it effects this is unknown, but it is interesting to note that when excessive doses of vitamin D are given under certain circumstances the plasma may become truly supersaturated to bone salt and a somewhat general calcification of the tissues may occur.

The chemical nature of vitamin D is unknown.[2] It is formed from ergosterol by the action of ultra-violet light, but nothing definite is known regarding the nature of the photochemical change that occurs in the sterol molecule.

In a recent attempt to isolate the vitamin from the product of irradiation of ergosterol, Askew, Bourdillon, Bruce, Jenkins and Webster (*Proc. Roy. Soc.*, 1930, **107**, *B*, 76, 91) have distilled the activated substance in a long narrow tube *in vacuo* and collected the fractions condensing at various temperatures. They find the part condensing at 100–110° the most active antirachitically, the parts condensing below this (90–100°) and above (110–120°, 120–130°, 130–140°) being much less active. From the most active fraction a further very active crystalline fraction can be separated after dissolving in alcohol containing a high percentage of water.

[1] For a full account of recent developments in this field consult Robison (*Biochem. J.*, 1930, **24**, 1905, 1922, 1927).

[2] Vitamin D has been isolated in what appears to be the pure condition by a group of investigators in England, and also by Windaus and co-workers in Germany. It is apparently an isomer of ergosterol. (*Proc. Roy. Soc.*, 1932, B, **109**, 488, *Annalen.*, 1932, **492**, 226.)

Further crystalline fractions cannot be obtained. There is no evidence that the crystals are pure vitamin D; the vitamin may be merely an impurity in this crystalline product. In a later paper they produce clear evidence that vitamin D is not the only product of the irradiation of ergosterol.

Vitamin E (Antisterility Vitamin).—This dietary factor is essential for the normal growth of certain species and for their normal reproduction. A characteristic form of sterility in which reabsorption of the foetus occurs as a result of a deficiency of this factor has been described by Evans and Burr (Vitamin E. *Memoirs of the University of California*. 1927. Vol. 8). Histological changes due to lack of vitamin E have also been carefully described by K. E. Mason in *Proc. Nat. Acad. Sci.*, **11**, 377, 1925, and in *J. Exp. Zool.*, **45**, 159, 1926. The richest sources of the vitamin are green leaves and the oil of wheat germ. It is comparatively stable, as it resists saponification of the oil and other severe chemical treatment, but nothing positive is known regarding its chemical nature.

GENERAL CONSIDERATION OF BIOLOGICAL TESTS FOR VITAMINS

Housing and Care of Animals

Generally speaking, it is convenient for rooms for the housing of small animals to be small; a convenient floor area is 12 feet square. If a large number of animals are to be accommodated several such rooms are preferable to one large one as it is then an easy matter to maintain different conditions in different rooms if it is desired. The separate small rooms are also a safeguard against the spread of infections which sometimes cause serious losses in laboratory animal colonies. The walls of the rooms should be covered with an impervious, washable surface, preferably white or pale yellow in colour. The floor must also be impervious and well drained so as to allow the whole surface of the room to be sprayed or washed down periodically. Adequate heating and ventilation must be provided, and automatic regulation of the temperature is in many cases desirable, particularly in rooms in which rats are kept. As far as possible, every fitting in the rooms should be of metal and their design should be such as to reduce to a minimum corners and crevices which might harbour pests, such as cockroaches and bugs. Each room should be fitted with a hot and cold water supply and a convenient sink.

The preparation of food mixtures should be made in a separate "kitchen," conveniently fitted with the necessary mixing appliances. stoves, etc.

The racks for carrying animal cages should be entirely of metal and should be so arranged that they can readily be moved and, if necessary, taken apart for thorough cleaning.

Periodically the rooms should be emptied, the surfaces washed down with disinfecting fluid and all equipment (cages, racks, etc.) sterilised, preferably by dry heat, before being brought again into use.

The rooms employed for housing rats for breeding or vitamin tests should be maintained at a temperature between 65 and 70° F. Guinea pigs are better kept at a lower temperature 55–60° F., whilst pigeons do not require any special regulation of their environment. The caging of all the species used for vitamin tests calls for special consideration. All cages must be of metal and it is essential that they should be fitted with false bottoms or gratings to permit the droppings to fall through. This is not only desirable on the grounds of cleanliness but it is an essential guard against the eating of excreta which sometimes occurs and which may profoundly disturb the results of certain types of vitamin test. Cages for stock pigeons should be sufficiently large to permit the birds to exercise by flight and the smaller cages for individual birds for vitamin tests should also be fitted with a metal perch. As each animal in a vitamin test must be kept in a separate cage, the type of cage used should be one that takes up as little space as possible and which it is easy to handle and clean.

A large number of cages can conveniently be arranged in rows on vertical racks, thus facilitating the task of feeding and cleaning. Clean sawdust is the best absorbent material for the trays to catch droppings, but several thicknesses of old newspaper or filter paper can also be used.

All animal cages should be cleaned out at least once a week, thoroughly scrubbed with soap and water, and periodically they should be washed with disinfectant solution and sterilised by dry heat at 120° C. Only by the strictest attention to cleanliness is it possible to maintain animal colonies reasonably free from parasites and epidemics. When infections do appear the affected animals must be immediately isolated and all cages, drinking vessels, etc. sterilised.

The following notes concerning the species usually employed for vitamin tests may be found useful, but only by long practice in the actual handling of the animals can the necessary experience and knowledge be gained.

(a) **Rats.**—It is in every way desirable that colonies of stock rats should be maintained from which may be derived young animals of known history for biological tests. Usually, either the albino or the piebald black and white variety of the rat, *mus norvegicus*, is used. The latter appears to be rather more vigorous than the white rat, but there are available pure strains of albinos of long established history,[1] about which a large amount of statistical information has been accumulated.[2]

The breeding stock can be fed on a variety of suitable food mixtures. Some laboratories use a very mixed diet, such as kitchen scraps, whilst in others a definite ration is employed.

It is advisable when rats are being bred for use in vitamin A and D tests to pay special attention to the diet of the breeding stock so as to supply sufficient of these vitamins for reproduction and the rearing of the litters, but not so much as to result in large stores being laid down in the tissues of the young animals. It is therefore of the greatest value to keep the diet of the breeding colony as nearly uniform as possible throughout the year.

Full details of types of breeding diets found to be suitable are given in the following papers.

Smith and Chick (*Biochem. J.*, 1926, **20**, 131); Sherman and others (*J. Biol. Chem.*, 1921, **46**, 503; 1922, **53**, 49); Gudjonssen (*Biochem. J.*, 1930, **24**, 1591); Coward, Cambden and Lee (*Biochem. J.*, 1932, **26**, 679).

Experience has shown, however, that a diet that has been found to be adequate in one laboratory is not always adequate in another. In view of the variations in some of the ingredients (*e. g.*, milk, fresh and dried), this is not surprising. It shows the necessity for each laboratory to work out for itself a suitable diet for its breeding colony from the materials available in its locality.

Drinking water is most conveniently provided from containers designed to prevent contamination. These should be refilled daily and cleaned and sterilised at least once a week.

[1] A strain of the albino *mus norvegicus*, originally bred and studied for many generations at the Wistar Institute, Philadelphia, is carried on in this country by the "Glaxo" Research Laboratory, 56, Osnaburgh Street, London, N.W.1. Animals for breeding and for vitamin tests may be obtained from this source.

[2] The rat (2nd Edition) Donaldson. Philadelphia, 1924.

Each pregnant doe should be given a separate cage and material—hay, cotton-wool or shavings, to build a nest. It is sometimes advisable to increase the vitamin B content of the diet of the mother during the period of lactation. This is best done by giving additional wheat germ or dried brewers' yeast. In some laboratories it is customary to reduce the number of young when large litters are born so as to leave the mother with not more than six or seven to rear. The young are usually weaned when 3 to 4 weeks old.

Every laboratory employing rats for growth experiments should prepare curves showing the normal growth of males and females of the stock to which can be referred, if necessary, the results of vitamin tests.

A colony of rats established in clean surroundings and fed on a balanced diet keeps remarkably free from disease. The chief danger is from an epidemic diarrhoea which seldom appears in healthy stock, but which may cause heavy losses in animals being fed on deficient diets. An outbreak may follow a fall in environmental temperature and it is difficult to save many of the affected animals. Isolation and rigorous sterilisation of cages and feeding vessels are the only practical measures that can be taken. Parasites may cause trouble unless the stock is kept under clean conditions. There is a rat bug, a rat flea and a rat louse, all of which flourish when wooden cages or fittings are used or when cleansing is inadequate. They affect the general health of the animals more than is sometimes appreciated. A form of scabies is often responsible for small warty growths on the tip of the nose and for small scaly areas on the ears, tail and legs. This disorder should never appear in a healthy colony, but if it does it can be readily treated by a few applications of sulphur ointment.

(b) **Guinea Pigs.**—It is advisable to breed guinea pigs to be employed for vitamin C work. They are easy to breed and the most convenient manner is to allow the stock to run in a large pen with a thick layer of sawdust on the floor and plenty of hay. A stock ration of oats and bran with liberal amounts of fresh green foods and roots will keep the animals healthy. The young are easily reared and are ready for use when they weigh about 250 gm.

(c) **Pigeons.**—Usually these are obtained from reliable dealers, as the difficulties of breeding the numbers required for vitamin studies have proved rather formidable. By keeping the animals under observation for a period after purchase, as recommended by

Peters (see p. 312), it is possible to select birds which appear to be healthy. A stock diet of mixed grain is suitable for this period, water being also provided.

2. Standards for Biological Assay of Vitamins[1]

It is very desirable in making an examination of foodstuffs for vitamins that the results should be expressed in terms which make it possible to compare them with the findings of another laboratory. This is becoming generally recognised at the present time and there is a definite move in the direction of securing international agreement both as to the methods of testing and as regards the terms in which the results shall be expressed. Meanwhile, a most confused and unsatisfactory state of affairs exists.

The nearest approach to a satisfactory method of assay is that recently adopted by the Medical Research Council and the Pharmaceutical Society for the estimation of vitamin D. In this test a comparison is made between the activity of the material under examination and that of a standard preparation of irradiated ergosterol (see p. 321). The results so far obtained with this method are definitely encouraging.

An essential for such a method is a stable preparation of the standard. It would appear that the irradiated ergosterol preparation now available in this country is sufficiently stable for this purpose when properly stored.

No such preparations are available yet as standards of reference for the assay of the other vitamins and the biological methods of assay must, perforce, be based for the time being on comparative tests of a much less clearly defined character.

ESTIMATION OF VITAMIN A

1. Biological Methods

Practically all the methods at present in use depend on observing the influence of the substance being tested on the weight of animals (usually rats) fed on a basal diet rendered as free from vitamin A as possible. The unsatisfactory character of a "growth test" is generally recognised but, as yet, no satisfactory alternative has been proposed as the basis of a biological method of assay of vitamin A.

[1] An important advance has been the attainment of international agreement on standards of reference for estimation of vitamins A, B_1, C & D This is fully described in Appendix I.

Methods of Assay of Vitamin A

(a) Hume and Smith
(b) Sherman and Stiebeling
(c) Drummond and Morton
(d) Coward, Key, Dyer and Morgan

(a) Method of Hume and Smith (*Biochem. J.*, 1928, **22,** 504) modified somewhat by Collison, Hume, Smedley Maclean and Smith (*Biochem. J.*, 1929, **23,** 634).

Young rats of 40 to 50 grm. weight are used, and the basal A-deficient diet is composed as follows:—

Purified caseinogen	300 grm.
Wheat starch	750 grm.
Irradiated hardened cottonseed oil	225 grm.
Salt mixture[1]	75 grm.
Dried yeast	255 grm.
Lemon juice	75 grm.
Distilled water	900 c.c.

Vitamin D is supplied by irradiating the melted hardened cottonseed oil in a thin layer for 30 minutes at a distance of 33 cm. from a quartz mercury vapour lamp.

Hume and Smith think that more reliable results are obtained by administering the foodstuff or other material to be tested for vitamin A from the start of the experiment than by observing the effects of administration on the animal after an initial period of depletion on the deficient basal diet. During the depletion period the health of the animals may suffer to a variable extent and the recovery when the supplement containing A is given may be considerably influenced by the extent of the previous injury to the tissues. The comparison is made on the basis of the observation that when the depletion period is omitted the weight curve flattens off after a period directly proportional to the size of the dose of vitamin A given.

When, however, the recovery type of experiment is performed, Hume aims at determining the dose of test substance which will just maintain weight in the animals used. Potencies of different substances are calculated from these doses.

[1] Salt mixture.

Sodium chloride	51.9 grm.
Magnesium sulphate	164.0 grm.
Sodium dihydrogen phosphate	104.1 grm.
Dipotassium hydrogen phosphate	286.2 grm.
Calcium phosphate	162.0 grm.
Calcium lactate	390.0 grm.
Ferric citrate	35.4 grm.

(b) **Method of Sherman and Stiebeling** (*J. Biol. Chem.*, 1930, **88,** 683).—Young rats from litters reared by stock fed on a ration of one third dried milk and two thirds whole wheat are separated at an age of three to four weeks and given a diet consisting of:

Purified casein	18 grm.
Salt mixture	4 grm.
Dried brewers' yeast	10 grm.
Sodium chloride	1 grm.
Corn starch	67 grm.
Irradiated commercial cholesterol	0.05 grm.

On this A-deficient diet the animals usually grow about 35 to 50 gms. before the decline sets in. They are then ready for use as test animals and are given supplements of the materials to be tested. A comparison is made on the basis of an increase of weight at the rate of 3 gms. weekly over a period of 5 to 8 weeks. The shorter period of 5 weeks is preferred (see below). The quantity of supplement which promotes growth at this rate is taken as containing 1 unit of vitamin A.

(c) **Method of Drummond and Morton** (*Biochem. J.*, 1929, **23,** 786).—The basal vitamin A-deficient ration is compounded from:

Purified caseinogen	20 grm.
Rice starch	72 grm.
Salt mixture	4 grm.
Yeast extract ("Marmite")	4 grm.

Vitamin D is supplied in the form of a dose of 0.01 mg. of irradiated ergosterol given twice weekly.

Young rats of 40–50 grm. weight are fed on this ration until their growth ceases and there is the characteristic decline. Groups are then fed on a range of doses of the substance to be tested and the effect on body weight observed over a period of 5 to 6 weeks.

Variation of Animals in Vitamin A Tests

All biological tests are subject to errors arising from the variation in the response of the individuals. The magnitude of these errors in the biological tests for vitamins is only slowly being appreciated, but it is now generally recognised that it may be particularly large in tests for vitamin A.

All investigators emphasize the importance of drawing the animals to be used for these tests from stock reared on a diet of definite composition. Vitamin A is stored in the tissues of animals fed on

normally balanced dietaries and only by maintaining a constant composition of the diet of the breeding stock (see p. 297) can one guard against the danger of serious errors arising from variations in the vitamin A reserves of the tissues.

When this precaution is taken the individual variation in response may still be considerable, even when the "recovery" test is employed.

Using groups of seven to twelve rats for each dose Drummond and Morton were unable to distinguish with certainty between two doses of cod-liver oil of which was less than 50% of the other (*Biochem. J.*, 1929, **23**, 785).

This important aspect of the assay of vitamin A has recently been examined in detail by Coward, Key, Dyer and Morgan (*Biochem. J.*, 1930, **24**, 1952, 1931, **25**, 551; and Coward (*Biochem. J.*, 1932, **26**, 691).

Basis of the Method of Coward, Key, Dyer and Morgan

Before June 1931 there was no general standard of reference for vitamin A. Therefore, at present, no absolute value can be given for the potency of any substance. It can only be stated in terms of the experience of the analyst who makes the examination and no result obtained in one laboratory can be interpreted by means of results obtained in other laboratories. It is, of course, possible for a laboratory to adopt its own standard of reference, determine the response to that standard under the particular conditions (*e. g.* animal, diet, temperature) of that laboratory and then express the results of an examination of a fresh sample in terms of the one used as a standard. This procedure has been recently adopted by the Pharmaceutical Society of Great Britain. A particular sample of cod-liver oil, considered by a preliminary test to be of good average quality, was submitted to a very thorough examination. Growth was taken as the criterion of vitamin A activity. Recognising the very varied response given by different rats to equal doses of vitamin A, it was decided to test different doses of cod-liver oil on different groups of rats which had ceased to grow on a shortage of vitamin A, each group consisting of about 30 rats and all rats in any group having equal daily doses. The average increase in weight of each group at the end of 5 weeks was then plotted against the dose and the resulting curve gave remarkably good agreement with the equation $y = 12.1 + 40.24 \log x$, where y = the average increase in weight (g.) of the group of rats in 5 weeks and x = the

daily dose of cod-liver oil in mg. The groups of 30 rats consisted of approximately equal numbers of bucks and does. When the average increases of the bucks in each group were plotted separately, they were found to give a curve represented by the equation $y = 11.3 + 50.3 \log x$. The does of the groups gave the curve $y = 12.4 + 27.4 \log x$. The range of doses used for the making of the curve was a very wide one. The lowest dose was 0.25 mg., the highest 7.5 mg.; thus the curve distinguishes between a range of doses of which the highest was 30 times the value of the lowest.

To estimate the vitamin A potency of a sample of cod-liver oil in terms of the sample used in making the curve, 10 or 12 animals which have ceased to grow on a shortage of vitamin A are all given an equal dose of the fresh oil. The average increase in weight of the group in five weeks is determined and, by reference to the curve, the dose of the standard sample of oil which would have given this average increase is found. Probably the most accurate result is obtained by the dose which gives an average increase of about 20 grams in 5 weeks. The inverse ratio of the dose actually given and the one of the standard corresponding to it gives vitamin A potency of the fresh sample relative to that of the standard. This method gives a result with a possible theoretical error of $\pm 30\%$. Actually on analysing the results obtained in the making of the curve it was found that the error obtained by the use of 10 animals was often considerably lower than this (cf. Coward *Biochem. J.*, [in the press]).

Obviously, the curve just described cannot be adopted without trial by any other laboratory. Differences in strains of rats (a mixed black and white strain was used for the curve), in the temperatures of different laboratories and, above all, in the basal diet used in the experiment may make the curve of response obtained in one laboratory quite different from one obtained in another laboratory.

It is claimed, however, that the smoothness of the curve obtained at the Pharmaceutical Society justifies the following conclusions. (1) "Litter mate" control is unnecessary, for each group of rats on one dose was completed as the animals became steady in weight: there was no attempt to make all groups contain members of the same litters. (2) There is no seasonal variation in the response of animals to vitamin A for the groups were not dosed in the order of the size of the doses, and the experiment extended over some eight months of one year.

(d) Details of the Method of Coward, Key, Dyer and Morgan. Rats should be selected from a colony fed on a diet only moderately rich in vitamin A. Some workers find it useful to give their breeding colony a rich diet, but at the beginning of the third week of lactation of a doe she is given the vitamin A-free diet. This is obviously a matter which must be determined in each laboratory.

Rats for a test should be given the vitamin A-free diet when they weigh about 40 grams. Their reserves should be such that they cease to grow when they have attained a weight of about 80–100 grms.

The vitamin A-free diet which was used in the work for the construction of the curve of response described above consisted of:

Caseinogen	15%
Dextrinised rice starch	63%
Dried brewer's yeast	8%
Salt mixture	4%

In addition, each rat was given about 2 units of vitamin D in oil twice a week.

The caseinogen was "light-white casein" (B. D. H.) extracted once with alcohol and 4 times with ether in the cold, each extraction being made for 24 hours with frequent shaking. Later work has shewn (*Biochem. J.*, 1931, **25**, 551) that this extraction is unnecessary, for rats which have ceased to grow on a diet containing the extracted casein do not resume growth when given a similar diet containing non-extracted casein; and, moreover, similar responses to a sample of cod-liver oil are obtained whether the test is made on a basal diet containing extracted light white casein or on one containing non-extracted light white casein.

The salt mixture (Steenbock's No. 40) consists of:—

$NaCl$	23.36 grm.
$MgSO_4, 7H_2O$	24.6 grm.
$Na_2HPO_4, 12H_2O$	35.8 grm.
K_2HPO_4	69.6 grm.
$Ca_2H_2(PO_4)_2, 4H_2O$	68.8 grm.
Ca lactate, $5H_2O$	15.4 grm.
Iron lactate, $6H_2O$	5.98 grm.
KI	0.16 grm.

The rice starch is partially dextrinised by mixing it with water to a very stiff paste and baking in broken lumps in an ordinary cooking-oven for about 6 hours. It is then coarsely ground.

Each rat should be weighed once a week for the first two or three weeks, but as the rate of growth lessens, weighings should be more frequent, at least 2 or 3 times a week. They should always be made at the same time of day. A rat may be considered steady in weight when it has not gained more than 2 grm. in 10 days. It may lose weight suddenly, and if it loses more than 12 grm. it will probably die even after being given a large dose of vitamin A. One fall in weight cannot be regarded as cessation of growth.

Expressing the Results of Vitamin A Assay

The method of Sherman and Munsell (*J. Amer. Chem. Soc.*, 1925, **47**, 1639) was made the basis of the official method of assay introduced into the 10th Edition of the U. S. Pharmacopoeia published in 1926. The step was premature, as neither method made allowance for the effects of vitamin D and both are now obsolete. They did, however, introduce the suggestion that a comparison of vitamin A activity might be made on the basis of the dose of foodstuff required to promote a rate of growth of about 3 gms. weekly for a period of not less than five weeks. This was regarded as the "unit" of vitamin A. Thus, a cod-liver oil which produced this rate of recovery in daily doses of 10 mg. would be regarded as containing 100 "units" of vitamin A per gm.

It has, however, been pointed out that estimations made on this basis are open to serious error (Drummond and Hilditch, Empire Marketing Board. Report No. 35, 1930, p. 25. See also *Biochem. J.*, 1930, **24**, 27) and that the use of the word "unit" is misleading in suggesting a degree of accuracy which is seldom reached. Moreover, the term "unit" as applied to biological assay should be reserved for that amount of activity contained in a given weight of a given preparation available as a standard of reference for all workers.

It would seem preferable to accept, for the time being, the method of comparison outlined on p. 302 and to work towards a method based on a reliable standard preparation such as has been adopted for vitamin D (see p. 321).

2. Spectroscopic Method of Assay

The true "vitamin A" is believed to be a colourless substance showing a characteristic absorption band in the ultra-violet with

a maximum intensity at about 328$\mu\mu$ (Morton and Heilbron, *Biochem. J.*, 1928, **22**, 987).

It has been shown by Drummond and Morton (*Biochem. J.*, 1929, **23**, 794) and by Coward, Dyer, Morton and Gaddum, (*Biochem. J.*, 1931, **25**, 1102) that in the examination of certain materials, *e. g.* fish liver oils, measurement of the relative intensities of absorption at this wave length gives figures in good agreement with the results of biological tests. The method is one of considerable promise, for the intensities can be measured to within 2% by the ordinary methods of quantitative spectrophotometry using the photographic technique, and to a definitely greater degree of accuracy using the photoelectric method. As yet the method has been employed chiefly for the examination of oils, but it is probable that it will prove to be of more general application. Some care is needed in the interpretation of bands in this part of the spectrum, as certain substances, *e. g.*, dehydro-ergosterol, may show an absorption in the same region as vitamin A (Rosenheim and Webster, *Biochem. J.*, 1929, **23**, 633). Morton, Heilbron and Spring (*Biochem. J.*, 1930, **24**, 136) have, however, shown that the absorptions can be differentiated.

3. Colorimetric Assay

Some years ago it was shown (Rosenheim and Drummond, *Biochem. J.*, 1925, **19**, 753) that substances containing "vitamin A" gave a blue-violet coloration with certain reagents, of which the most promising appeared to be arsenic trichloride. The test was greatly improved by the modifications introduced by Carr and Price (*Biochem. J.*, 1926, **20**, 497), the chief of which was the substitution of antimony trichloride for the corresponding arsenic compound.

As is now generally recognised, the test is in many respects far from ideal as the basis of a method of assay of vitamin A. In the first place it is not strictly specific for the vitamin. Certain other substances, particularly the pigments of the carotene class, carotene, xanthophyll, crocetin, etc. give blue colours similar to the eye to that produced by substances containing the vitamin. Furthermore, the intensity of the colour is dependent on a number of factors, of which the more important are the dilution of the substance being tested, the concentration of the solution of antimony trichloride and the time elapsing between mixing the reagents and measuring the colour produced.

The influence of these factors has been studied in some detail by Wokes and Willimott (*Biochem. J.*, 1927, **21**, 419; *Analyst*, 1927, **52**, 515), who made the following suggestions regarding the application of the reaction to the quantitative estimation of vitamin A in such materials as fish-liver oils.

(a) *Reagent.*—A saturated solution of pure antimony trichloride (recrystallised from chloroform), concentration 26 or 27%, weight in volume, in anhydrous chloroform. The reagent should be protected from light and moisture, and decanted before use. Two c.c. to be placed in a clean dry cell $\frac{1}{2}$ in. deep (thickness).

(b) *Solution of Oil.*—To be prepared freshly with anhydrous chloroform. Strength to be adjusted so as to keep the amount to be added between 0.1 and 0.3 c.c. This to be run into the cell already in position in the tintometer, and containing the reagent. To be mixed immediately by stirring with a clean glass rod, and the time of mixing noted.

(c) *Tintometer Readings.*—To be measured in Lovibond units (blue), 30 seconds after mixing. Conditions to be adjusted so that reading of blue units is below 18.

(d) *Temperature.*—To be kept at about 16° C. Divergence of more than one degree from this to be noted, and a correction applied.

In a more recent study of the conditions of applying the test to liver oils, Norris and Church (*J. Biol. Chem.*, 1929, **83**, 469; 1930, **87**, 139) recommend the use of a chloroform solution of antimony trichloride prepared as follows:—

Thirty grm. of fresh Baker's analysed antimony trichloride in 100 cc. chloroform at room temperature. Cooled to 2–4° before being used. Then contains about 18% $SbCl_3$ (weight volume) "thus permitting the use of lower temperatures than room temperature under ordinary laboratory conditions, for a long enough period to obtain an accurate reading."

The method has been most widely studied in relation to the examination of products, such as the fish liver oils, which are relatively rich in vitamin A and which can conveniently be subjected to the test. Various techniques have been proposed. Rosenheim (*Biochem. J.*, 1927, **21**, 1329) gives the following details:

By means of a standard pipette 1 c.c. of the oil is measured into a measuring flask of 5 c.c. capacity. The pipette is rinsed out three times with chloroform, and the solution made up to volume

with the same solvent. Of this solution 0.2 c.c. of the solution is measured into a test tube (10 mm. diameter) and mixed with 2 c.c. of a 30% solution of antimony trichloride in chloroform, delivered from a standard burette. The blue colour developed on mixing is measured in a Rosenheim-Schuster pattern of the Lovibond Tintometer within a minute of mixing.

An alternative technique (Drummond and Hilditch. *E. M. B. Report*, No. 35, 1930, p. 32) recommends a 10% solution of the oils in chloroform:—About 1 grm. of the oil is accurately weighed out in a 10 c.c. volumetric flask and dissolved in 10 c.c. of redistilled chloroform. The temperature of the solution must be between 15 and 17° C., as must also be that of the antimony trichloride reagent. Into a glass cell of 1 cm. internal thickness there is pipetted 0.2 c.c. of the oil solution and to it there is quickly added from a burette exactly 2 c.c. of a 30% solution of antimony trichloride solution in chloroform. The cell is gently shaken during the addition of the reagent and exactly 30 seconds after the addition was begun the blue colour is matched in a colorimeter of the Rosenheim-Schuster pattern. Only the measurement of the blue colour need be noted, and it is most conveniently expressed in terms of the standard Lovibond tinted glasses. It is best to employ a source of diffuse daylight from a north window transmitted through the special Lovibond paper. At least three separate estimations should be made.

If the colour produced in this test by a sample of oil is more than 10 blue Lovibond "units," a set of estimations must be made on lower concentrations; on the other hand, if the response is not so good, higher concentrations should be tested. The results are plotted and the value for a 10% solution read from the chart, if the relation is linear. If it is not, as is occasionally the case with oils of low grade, it is advisable to separate the non-saponifiable matter with precautions against oxidation and carry out the test on appropriate dilutions of that material. The interfering substances are removed by saponification, and much better results may then be obtained.[1]

The chief difficulty in applying the method to the quantitative estimation of vitamin A is that the relation between the concentra-

[1] Recent work indicates that the determination of the "Lovibond blue" on the unsaponifiable fraction yields results which are in closer agreement with the biological tests than those obtained by examination of the oils themselves.

tion of vitamin and the colour produced is frequently not linear. In the case of cod-liver oils it has been shown (Norris and Church. *J. Biol. Chem.*, 1930, **85**, 477; Drummond and Hilditch (*loc. cit.*)) that secondary reactions involving the unsaturated fatty acids may be the cause of this departure from the linear relationship. For this reason it is advisable to make comparisons only at dilutions at which it is known that the linear relationship holds. If this cannot be conveniently done, it is necessary to subject the material to saponification and carry out the colorimetric determination on the non-saponifiable fraction, for which it has been established that there is in general a linear relation between the intensity of the blue colour and the concentration of material taken.

Methods for the application of this modification of the test have been described by various investigators (Drummond and Hilditch. E. M. B. Report, No. 35, 1930, p. 33; Norris and Church. *J. Biol. Chem.*, 1930, **87**, 139, 477; Smith, *Analyst* 1931, **56**, 9).

A more accurate method of estimating the blue colour than by the Lovibond tintometer is to employ the Hilger-Nutting spectrophotometer. This has been described by Drummond and Morton (*Biochem. J.*, 1929, **23**, 792). It has the advantage of enabling one to measure the intensity of the absorption with a maximum at 572μμ, which is believed to be the blue colour produced by the vitamin with antimony trichloride.[1]

[1] An important observation on the blue colour reaction for vitamin A has recently been reported by Gillam and Morton (*J. Soc. Chem. Ind.*, 1931, **50**, 440). They have found that liver oils contain two chromogens which give blue colorations with antimony trichloride, with maxima at 606 μμ and 572 μμ respectively. In concentrates these maxima are displaced to 620 and 583 μμ. A comparison of these colours with the ultra-violet absorption indicates that the latter band shows a definite parallel with the 328 μμ band, but that a similar relation between the 606 and the 328 μμ bands is not always found. This suggests that the matching of blue colours produced by antimony trichloride against Lovibond glasses may serve as a rough guide to vitamin A potency, but that it is theoretically unsound. Recent work by Coward, Dyer, Morton and Gaddum (*Biochem. J.*, 1931, **25**, 1102) on eleven samples of cod-liver oil and two concentrates indicates, from a statistical computation of the results, that there is a quite definite correlation between the vitamin A value of the oil measured biologically and both the 328 μμ absorption band of the oil itself and the Lovibond measure of the blue colour obtained on treating the unsaponifiable fraction of the oil with antimony trichloride. There was a significant correspondence, also, between these values of the spectrometric measurements of the 572 μμ and 606 μμ bands of the blue colour obtained with $SbCl_3$ on the oil itself, but in view of Morton's finding that these two bands do not always have the same relative value in different oils, it would be unwise to stress their apparent correlation in this small number of oils.

The blue value obtained by the Lovibond measure from the action of antimony trichloride on the oil itself showed definitely less correlation with the vitamin A value. Indeed, the chance that the 328 μμ band is more significant in the measure of the vitamin A value of an oil than the Lovibond blue measure of the oil is rather more than 0.95; while the chance that the Lovibond blue measure from antimony trichloride in the unsaponifiable fraction of the oil is more significant than that on the oil itself is about 0.8.

There was definite evidence also of the probable existence in certain oils of some substance which enhanced the blue colour obtained with antimony trichloride and in other oils of some substance which tended to inhibit the formation of the blue colour. Otherwise it would appear that some hitherto unsuspected factor had influenced the biological test of these particular samples.

The great promise of spectrometric methods in this direction s very clearly demonstrated by the recent paper of Heilbron and Morton *on the vitamin content of butter* (*Biochem. J.*, 1930, **24**, ;70). Butter contains both carotene and vitamin A, and it was ound possible to estimate both with some degree of accuracy by pectroscopic methods.

The antimony trichloride colour reaction has not yet been applied ;enerally to the examination of foodstuffs for vitamin A. It cannot, f course, be directly applied to any products which contain carotene r other substances giving blue colorations similar to that given by vitamin A." It is, therefore, of use mainly in the examination of oodstuffs of animal origin which contain little or none of these 'isturbing substances; indeed, it would appear that its utility will e confined almost entirely to the examination of fish liver oils and imilar products.

ESTIMATION OF VITAMIN B_1

Numerous methods of assaying foodstuffs for vitamin B_1 have een described. They can be divided into two groups according o whether a restoration of growth or a cure of the so-called "polyeuritis" is taken as a criterion of the presence of the vitamin. :ither rats or pigeons are convenient for the tests.

Assay by Comparison of Rat-growth Method of Chick and Roscoe *Biochem. J.*, 1929, **23**, 498).—Young rats of 40–50 gms. in weight re fed on one of the following diets.[1]

E.L.	Coagulated egg white[2]	100 grm.
	Water	700 grm.
	Rice starch	300 grm.
	Cottonseed oil	75 grm.
	McCollum's salt mixture (185)	25 grm.
	Distilled water	50 grm.

Supplement 0.05 to 0.1 grm. of cod-liver oil daily, according to size of the animal.

P_2L	Purified caseinogen	100 grm.
	Rice starch	300 grm.
	Cottonseed oil	75 grm.
	McCollum's salt mixture (185)	25 grm.
	Distilled water	500 grm.

Supplements 0.05 to 0.1 grm. of cod-liver oil daily, together with 0.4 grm. of utoclaved yeast as a source of B_2.

[1] It is recommended that both diets be "cooked" by steaming for 3–5 hours to prevent the :currence of "refection."

[2] The egg white protein is prepared by coagulating fresh white of eggs in a double saucen. The coagulum is put through a mincing machine and mixed with the dry constit:nts of the diet. This preparation supplies adequate amounts of B_2 vitamin.

Fed on either of these diets the young animals cease to grow after about 2 weeks, decline and die in three to four weeks unless the deficiency of vitamin B_1 is made good.

A comparison can be made between the quantities of foodstuffs required to restore a rate of growth of from 10 to 12 g. a week.

Method of Evans and Lepkovsky (*J. Nutrition*, 1929, **2**, 1).—These investigators stress the importance of having a uniform basal diet when comparative studies of the antineuritic vitamin B_1 are made.

They recommend a simple type of artificial ration:—

Casein (purified)[1]	45 grm.
Sucrose	45 grm.
Salts	4 grm.
Autoclaved yeast[2]	10 grm.
Cod-liver oil	2 drops daily.

[1] Commercial casein (Golden State Milk Products Co.) was precipitated from milk with hydrochloric acid. The commercial product was extracted in wooden tubs with acidulated water (40 c.c. glacial acetic acid to 40 l. of filtered tap water) by changing the water twice daily for six days. On the seventh day the casein was extracted twice with distilled water. After draining it was treated once with 50–80% alcohol and once with 85–95% alcohol, followed by one extraction with ether. It was dried at room temperature under a current of air from a fan.

[2] Whole dried bakers' yeast. (Fleischmann Co. N. Y.) Autoclaved for 5 hours at 18–20 lbs. pressure in layers less than 1" in depth.

On this diet the rats show a cessation of growth at about 80 grm. weight and then decline until death intervenes, usually with symptoms of "beri-beri." These investigators prefer the fat-free diet described above, as the presence of fat (10–50%) in the basal ration tends to make the animal less sensitive to the effects of B_1 deprivation.

Method of Smith (*U. S. Pub. Health Service.* Report No. 1348, 1930).—This method is based upon the production of "polyneuritis" in rats on a diet deficient in B_1:—

Casein	18%
Salt mixture	4%
Autoclaved brewers' yeast	10%
Cod-liver oil	1%
Olive oil	9%
Corn starch	58%

The substances to be tested are administered by intravenous injection, and the assay is based on the determination of the minimum curative dose.

Curative Test on Rat "Polyneuritis"

Method of Kinnersley, Peters and Reader (*Biochem. J.*, 1930, **24**, 1820).—Adult albino rats (males about 250 g., females about 170 g.) are fed on the basal diet:—

Caseinogen	20 parts.
Rich starch	70 parts.
Agar-agar	2 parts.
Salt mixture	5 parts.
Cod-liver oil	3 parts.
Alkaline autoclaved yeast extract[1]	6 parts.

[1] For preparation of this supplement providing B_2 see p. 317.

After about 21–24 days, during which considerable loss of weight occurs, the animal exhibits, often quite suddenly, characteristic convulsive symptoms. As soon as these are marked the test substance is given in measured amount to the animal by the mouth.

An improvement may often be noticed, if the substance given contains B_1, in as short a time as 20 minutes after dosing. Another attack of the convulsions follows when the effects of the dose have passed. The number of days between the administration of the dose and the recurrence of symptoms is noted, and the "day-dose" is calculated by division. This method is particularly useful in assaying the potency of liquid concentrates of B_1. It gives results which are in good agreement with the curative pigeon test (*vide infra*) but it is not suitable for the examination of foodstuffs as the animals in the convulsive stage are usually unable to eat food that may be offered to them.

Curative Test on Pigeon Polyneuritis

Method of Kinnersley, Peters and Reader (*Biochem. J.*, 1928, **22**, 276).—The pigeons are kept for 20 days or a month after receipt from the dealer on a mixed diet of buckwheat 1 part, dari 1 part, split maize 1 part, split wheat 1 part, wheat 2 parts.

If they appear to be normal birds at the end of this period of observation they are given a diet of Patna rice which has been washed for 3 hours in running water, re-dried, and autoclaved at 120° for 2 hours. The environmental temperature of the experimental birds should preferably be about 10–15° C.

When symptoms of "polyneuritis" appear the birds are brought into a warmer environment (*circa* 20° C.) and at this stage it is necessary to be on the guard against what are described as "heat cures."[1]

Only birds showing the classical symptoms of head retraction before 30 days should be used. They are first dosed with 50 mg.

[1] For a full description of this form of disturbance the original paper by Kinnersley, Peters and Reader should be consulted.

of glucose in 5 c.c. of water, and kept in a warm place for 2 to 3 hours to eliminate the various sources of extraordinary cure. If no cure occurs the bird is suitable for use as a test animal. The dose is delivered into the crop by means of a pipette fitted with a short length of bicycle-valve tubing. If the symptoms are relieved by the material given, a note is made of the time elapsing between the administration and the recurrence of head retraction. The dosage divided by the number of days of freedom from symptoms gives the "day dose" and this forms a basis for the comparison of the B_1 activities of preparations. The meagre observations of Kinnersley, Peters and Reader show that there is a quantitative relation between the time for which protection is given and the curative dose administered, provided that short periods of protection (*e. g.* under 10 days) are considered.

This method is particularly useful for the examination of water-soluble concentrates of vitamin B_1. It is unsuitable for the examination of foodstuffs owing to the inability of the pigeon to consume food during the convulsive stage of the disorder.

ESTIMATION OF VITAMIN B_2

Method of Chick and Roscoe (*Biochem. J.*, 1928, **22**, 790).—Young rats of 40–50 g. weight are given a diet composed of the following constituents:

Purified caseinogen[1]	100 grm.
Rice starch	300 grm.
Cotton seed oil	75 grm.
McCollum's salt mixture (No. 185)	25 grm.
Water	500 c.c.

(the diet is heated, before use, for 3 hours in steam at 100°, in order to prevent the appearance of "refection" in the experimental animal).

[1] The caseinogen is "light white soluble" and is purified by precipitation from a 5 % solution by acetic acid, washing with 0.05 % acetic acid twice a day for a fortnight, extracted for 96 hours with 50–70 % acidified alcohol (0.03 % acetic) in a Soxhlet, dehydration with 93 % alcohol, drying at low temperature and heating at 120° in a finely ground condition for 3 days.

The diet is supplemented daily with 0.05 to 0.1 grm. of cod-liver oil, according to the size of the rat, and a supply of Peters' concentrate of B_1 (see p. 317) equivalent to 0.6 grm. of dried yeast. On this diet the animals tend to show a loss of weight, although, in some cases, the decline may be several weeks in appearing. The skin symptoms, dermatitis and loss of hair, are usually bilaterally symmetrical and appear at any time after the 5th or 6th week.

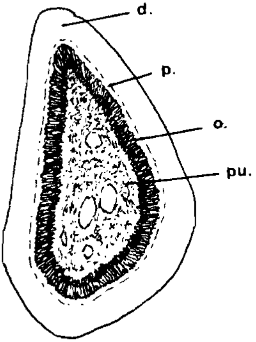

FIG. 1.

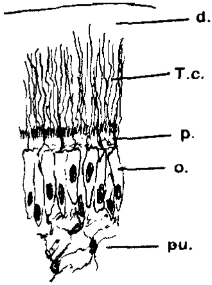

FIG. 1A.

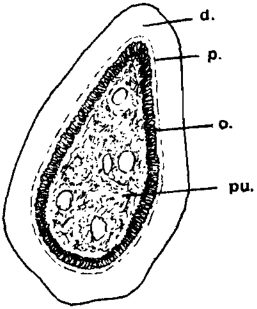

FIG. 2.

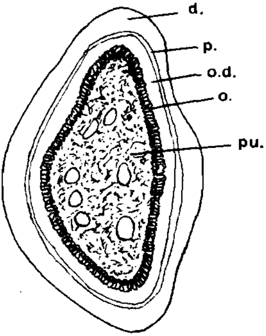

FIG. 3.

EXPLANATION OF PLATES.

FIGS. 1–3.—Progressive histological changes in the teeth of guinea-pigs fed on a scorbutic diet: d, dentive; p, predentive; o, odontoblasts; pu, pulp; T.c., Tomes canals; o.d., osteodentine; ost, osteoblasts.

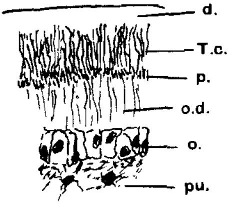

FIG. 3A.

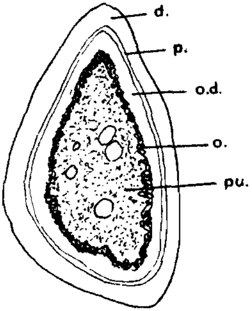

FIG. 4.

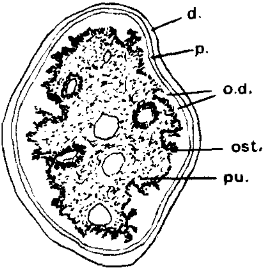

FIG. 5.

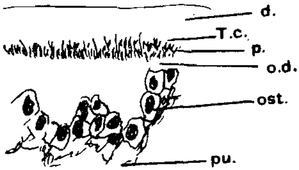

FIG. 5A.

EXPLANATION OF PLATES.

FIGS. 3A–5A.—Progressive histological changes in the teeth of guinea-pigs fed on a scorbutic diet: d, dentine; p, predentine; o, odontoblasts; pu, pulp; T.c. Tomes canals; o.d., osteodentine; ost, osteoblasts.

The symptoms have been fully described by Chick and Roscoe (*loc. cit.*), by Findlay (*J. Path. Bact.*, 1928, **31**, 353) and by Goldberger and Lillie (*U. S. A. Public Health Reports*, 1926, **41**, 1025).

After 3 to 4 weeks from the beginning of the feeding on the deficient diet the young rat is in a fit state to be used as a test animal for B_2 preparations. A convenient basis for comparison is the weight of material which gives an average weekly increase in weight of from 11–14 grm.

Curative Test on Dermatitis.—Instead of judging the values of foodstuffs as a source of B_2 by the recovery of weight of the animal, a comparison can be made of their efficacy in dispelling the characteristic skin symptoms of B_2 deficiency. This is, however, a more tedious procedure as it is necessary to maintain the rats for a longer time on the basal ration for the appearance of the symptoms than for the decline of weight. Roscoe (*loc. cit.*) finds a general agreement between the results of the two tests for B_2, but thinks that less of the active substance is needed to cure the dermatitis than to restore a good rate of growth.

ESTIMATION OF VITAMIN B_3[1]

Method of Eddy, Gurin and Keresztesy (*J. Biol. Chem.*, 1930, **87**, 729).—Pigeons are fed on a diet of polished rice until the characteristic symptoms of failure of nutrition due to B_1 deficiency are well advanced. They are then given a preparation of B_1, preferably as a concentrate such as that described on p. 317, in sufficient amount to prevent the appearance of "polyneuritis" (head-retraction, convulsions, etc.). After about 10–14 days on the supplemented basal diet, during which there is no recovery of weight, the foodstuff to be tested is administered in weighed daily doses and the effect on the weight of the birds observed. The extent of the recovery of body weight can be employed for comparing the relative value of the supplements as sources of B_3.

ESTIMATION OF VITAMIN B_4

Reader (*Biochem. J.*, 1930, **24**, 1827) has recently described a method for the detection of this factor. Rats are employed. The animals are fed on the following basal diet:—

[1] Williams and Waterman, *J. Biol. Chem.*, 1928, **78**, 311.

Caseinogen (free from A, D, B_1 and B_2)[1]	20%
Rice starch	70%
Agar agar	2%
McCollum's salt mixture[2]	5%
Cod-liver oil	3%

[1] Obtained from Messrs. Glaxo Ltd.
[2] See p. 300.

Each rat receives supplements of vitamins B_1, B_2 and B_4 sufficient to permit normal growth to maturity.[1]

B_1 Supplement.—The concentrates used are the 0.1*N* HCl or 50% alcohol extracts from charcoal. In each case they are fractionated into 99% alcohol after removal of metals by H_2S. (See Kinnersley and Peters, *Biochem. J.*, 1928, **22**, 425.) Two to three pigeon-day doses of this concentrate are given daily to each rat.

B_2 Supplement.—Yeast extract heated to 120° for one hour at *p*H 9. After being carefully neutralised it is mixed with the basal diet to correspond with an addition of 6% of the original extract. (Reader, *Biochem. J.*, 1930, **24**, 77.)

B_4 Supplement.—Wash the mercuric sulphate precipitate from the Kinnersley and Peters process of concentration of B_1 with distilled water (*Biochem. J.*, 1927, **21**, 777) until washings give a blue colour with bromphenol blue. Suspend the precipitate in water and decompose with hydrogen sulphide. Filter off the mercuric sulphide, remove hydrogen sulphide by distillation in vacuo 40–60°. Bring to *p*H 3 with sodium hydroxide. Concentrate to a volume corresponding to 5 c.c. for each 100 grm. of original yeast, adjust *p*H to 4.5 and add alcohol to 50%. Cool overnight, filter off any solid, remove alcohol *in vacuo* and concentrate. Add acetone to 80% and again stand overnight. Decant and remove acetone at low temperature. Make final preparation up to a volume 1 c.c. equivalent to 100 grm. original yeast. The daily dose for a rat varies from 0.25 to 0.05 c.c.

The fully-grown animals are then deprived of the supplements of B_1 and B_4. The animals decline in weight rather sharply, and after about four weeks convulsions occur accompanied by great weakness and ataxia. The true B_1 deficiency symptoms are then dispelled by the administration of sufficient B_1 supplement, leaving the animal in a condition that is believed to be due to the absence of B_4 alone. The weight ceases to fall, but the animal shows a lack of co-ordination and walks with a rolling motion. If a foodstuff containing B_4 is given the weight soon increases and within a few days the locomotion of the rat becomes normal.

ESTIMATION OF VITAMIN C

The juice of fresh lemons has been found to be of such constant antiscorbutic potency that it is generally accepted as a provisional standard of reference for other substances. Some investigators prefer to remove the greater part of the organic acids by adding an excess of chalk and filtering off the precipitate before administering to the guinea pigs, but it does not seem to be essential.

[1] The rat appears to be able to thrive without the factor B_3 found necessary for pigeons.

The juice, whether "decitrated" or not, should always be freshly prepared.

Höjer's Method.—Some years ago Zilva and Wells (*Proc. Roy. Soc.*, 1919, **90**, B, 505) drew attention to characteristic lesions in the teeth of guinea-pigs with scurvy. This observation has been made the basis of a method of assay by Höjer (*Acta pediat.*, 1924, **3**, (*Suppl.*); *Brit. J. Exp. Path.*, 1926, **7**, 356):—

His basal scorbutic ration is:—

Bran	45 grm.
Crushed oats	25 grm.
Dried separated milk	30 grm.

Supplemented by 1 c.c. of cod-liver oil twice weekly.

As animals vary in their response to this diet, it is necessary to determine by a series of experiments the degrees of scurvy which can be distinguished and also to ascertain the number of animals that are required to give an average response to a given dose of antiscorbutic vitamin under the conditions prevailing in the particular laboratory.

When these observations have been made, the substance to be tested is administered in a range of doses chosen with regard to its probable activity. Each group should consist of not less than three animals. A dose of 3 c.c. lemon juice is required in this test for complete protection.

When the approximate potency has been judged, a narrower range of doses can be tested to determine the curative dose more accurately.

The animals are killed at the end of 14 days' feeding on the basal diet and the various supplements. The lower jaw bone of each is removed and placed in about 100 c.c. of Jenkins' decalcifying solution, which consists of:—

Concentrated hydrochloric acid	4 c.c.
Glacial acetic acid	3 c.c.
Chloroform	10 c.c.
Water	10 c.c.
97 % alcohol	73 c.c.

About 7–10 days are required for decalcification. A small piece of the jaw bone containing the root of one of the incisors at the region of the angle of the bone is cut out, embedded in gelatin and transverse sections cut by a freezing microtome. These are

mounted in a thin layer of gelatin on a microscope slide, stained with Hansen's haematoxylin and eosin.

The histological changes in the structure of the roots of the incisors should be judged according to:—

	Normal tooth	Scorbutic tooth
1. Dentine	Wide.	Narrow.
Tomes canals	Extend from odontoblasts through the dentine.	Wider than in normal tooth.
2. Predentine	Narrow and uncalcified.	Calcified and collagen-free.
3. Pulpa-bone or inner dentine	None.	Wide band, even projecting irregularly into the pulp. Tomes canals absent.
4. Odontoblasts	Long, parallel.	Wholly disorganised.

These four structures may not be equally disturbed in the same tooth. For the most accurate estimate of the degree of scurvy produced in an animal, each structure should be assessed independently and a general average of the results made for each guinea pig. The average of the animals on each dose will then give the value for that dose.

The correlation between the degree of scurvy and lack of vitamin C, however, has not yet been definitely established; it is therefore necessary to determine the minimum dose of test substance that prevents disturbance of the structure of the teeth. The inverse ratio of this dose to the curative dose of lemon juice is taken as the vitamin C content of the test substance relative to that of lemon juice. A series of characteristic conditions of the teeth is shown in Figures 1–5.[1]

Zilva's Method of Assay of Vitamin C.—Another method of testing for vitamin C in foodstuffs is that used by Zilva.[2] It depends on the degree of protection from scurvy which the substance tested affords to young guinea-pigs subsisting on a scorbutic diet. The animals should weigh 250–300 grm. at the beginning of the test.

The scorbutic diet given to the animals consists of:—

Bran	6 parts by volume.
Barleymeal	2 parts by volume.
Middlings	3 parts by volume.
Fishmeal	1 parts by volume.
Crushed oats	4 parts by volume.

[1] We are indebted to the Editor of the *Quarterly Journal of Pharmacy* for permission to reproduce these drawings. This method has recently been studied by Key and Elphick (*Biochem. J.*, 1931, **25**, 888).

[2] This method is fully described in *Medical Research Council Special Report*, No. 146, 1930.

Each guinea-pig is given, in addition, 40–60 c.c. of autoclaved milk made up from a dried milk powder each day. Guinea pigs of 250–300 grm. initial weight succumb to scurvy on this diet in 4–5 weeks.

The test can be carried out with or without a preparatory period of 14 days' feeding on the scorbutic diet alone. When foodstuffs of low antiscorbutic value are to be tested, it is desirable to omit the preparatory period, and carry out the test purely as a prophylactic one. When substances of higher value are to be tested, the preparatory period may be introduced.

Sixty days is often considered long enough for a test of this kind. To make it more stringent it may be carried on for ninety days.

Animals given the scorbutic diet alone lose weight rather rapidly and die with symptoms of acute scurvy in from 3 to 5 weeks. Animals given a small dose of vitamin C may grow for a short time but eventually become steady in weight or may lose weight and die. Animals given an adequate dose of vitamin C will grow as much as 250 grams in 90 days. Thus the growth alone may, with a large enough number of animals on each dose, give a fair idea of the potency of each dose. But signs of scurvy should also be determined. The animals must therefore be killed at the end of the experiment and examined for haemorrhages at the knees and in the peritoneum and gums, looseness of teeth, "white lines" at the costochondral junctions.

The dose of test substance which has given growth approximately equal to that of the animals given the lemon juice and which has protected the animals from scurvy may be said to contain as much vitamin C as 1.5 c.c. lemon juice. The antiscorbutic potency of the test substance relative to that of lemon juice is in the inverse ratio of the dose of test substance giving protection to the dose of lemon juice (1.5 c.c.) which gave protection and may be stated as a fraction or a percentage of the latter.

ESTIMATION OF VITAMIN D

The vitamin D content of a substance is estimated by comparison with that of a particular preparation of irradiated ergosterol issued by the National Institute for Medical Research as a standard of reference. The following statement was made by the Medical Research Council in August, 1930. It was published in the British

Medical Journal, the Lancet, and the Pharmaceutical Journal, August 30th, 1930.[1]

A Standard for the Antirachitic Vitamin D

Statement by the Medical Research Council.—The Medical Research Council's Committee on Accessory Food Factors have long attempted to find generally acceptable conventions for the quantitative record of the activities characteristic of the different accessory factors, or so-called "vitamins." As in other instances, however, any unit of activity based on the production of a stated degree of a biological effect, however carefully this may be defined, must be liable to wide variation with differences in the conditions under which the test is conducted. This is especially so in testing for a vitamin action where the effect on which measurement is based is the correction of an abnormal condition produced by a deficient diet. The severity of the condition to be treated will vary with differences in the dieting and treatment of the experimental animals, as well as with racial and individual differences in the stocks. Other biological standards, such as those for sera, hormones, etc., held by the Council's Department of Biological Standards, are based on stable standard materials, in terms of each of which a unit of activity is defined which is independent of the details of the biological method used in its measurement. Here a measurement of activity in units is made on a strictly comparative basis, with reference to the standard.

With regard to one of the vitamins, the antirachitic vitamin known as Vitamin D, this necessity for a common material standard was recently emphasised in the following recommendation by the Committee:—

> "Any unit or standard based directly on the response of the experimental young rats to administration of antirachitic material is unsatisfactory, owing to the disproportionate variation in the response to equal doses of apparently similar animals when tested at different seasons of the year or bred in different laboratories. The Vitamin D unit should rather be referred to a stated quantity of some standard antirachitic material, with which comparison can be made by any reliable biological method."

The discovery of the method of producing this vitamin artificially from ergosterol has led to its manufacture on a large scale, its issue in many forms for therapeutic use, and its addition to various

[1] We are indebted to the Medical Research Council for permission to reproduce this important statement.

types of food materials. The proper desire of manufacturers to use and to supply such preparations with a quantitative indication of activity has led to the appearance of several unofficial "units," mostly defined in terms of some biological effect, and having no clearly determined relation to one another. The need for common basis of measurement is therefore accentuated, in this case, by an existing and growing confusion. One of the provisional units current in this country, namely, that adopted by the Pharmaceutical Society's Laboratories when reporting on the vitamin D content of materials submitted to them for assay, has, indeed, been based on a material standard consisting of an oily solution of irradiated ergosterol, supplied to them for this purpose by the National Institute for Medical Research. Only a small quantity of this material had been prepared, however, and sufficient data were not available to justify the assumption that it would have the stability necessary for a standard and allow the Committee to recommend it for general use. It was accordingly decided to arrange for the preparation by the National Institute of a similar solution, on a scale sufficient for its eventual distribution to the numerous laboratories or institutions likely to require it, and to organise an investigation of its properties by different experts working on behalf of the Committee. A quantity of ergosterol from yeast, purified and dried by methods carefully recorded, was subjected, in an accurately made alcoholic solution, to irradiation with the rays from a mercury arc, the physical details of the procedure being measured and recorded with the greatest practical accuracy. All conditions known to influence the amount of vitamin D formed should, therefore, be reproducible at any future date, or in any other laboratory, leaving only the final adjustment of a new standard to equivalence with this original one to be made on the basis of comparative biological assay. The irradiation products were carefully freed from alcohol, dissolved in pure, unadulterated olive oil, and the volume adjusted to produce a known concentration, in terms of ergosterol used. The solution was then placed, and its main bulk has since been kept, in the Institute's refrigerating chamber at a temperature of $-4°$ to $0°$ C.

Evidence of the stability of such a preparation, under different conditions and over a sufficiently long period, being of central importance for a decision as to its use as a standard, the investiga-

tions promoted by the Committee have of necessity been of a corresponding length. The experimental results from the different contributing laboratories have recently been completed, and have now been carefully reviewed by the Committee, who have stated their conclusion from them in the following terms:

"From these tests it appears that specimens of irradiated ergosterol, prepared in the manner described above, do retain their activity unchanged for two years if maintained at or below 0° C. If they are kept at room temperature loss of activity may occur."

The evidence of the different investigators being uniformly favourable as to the suitability of this solution in other respects for use as a standard in comparative biological measurement of *Vitamin D, the Committee decided to recommend its adoption* as a standard, and the definition of a unit in terms of it, and adopted the following resolutions:—

"(*a*) That the standard solution of irradiated ergosterol prepared and maintained by the National Institute for Medical Research be recommended for adoption as a standard for the comparative estimation of Vitamin D.

"(*b*) That the unit of Vitamin D be defined as the antirachitic potency of a quantity of this preparation corresponding to 0.0001 mg. of the ergosterol used in its production.

"(*c*) That either the X-ray method, the Line test, or chemical analysis of the bones of the experimental animals be recommended for use in the estimation of Vitamin D.

"(*d*) That supplies of the standard material be made available for general distribution from the National Institute for Medical Research, Hampstead. As the supply is limited, it is hoped that users will themselves prepare standard solutions of irradiated ergosterol for use in individual tests, retaining the National Institute's material for reference. It is important that all such standard materials be kept at temperatures not exceeding 0° C."

It may be mentioned that the unit recommended has the same value, in terms of the weight of the original ergosterol represented, as that used for some years past in the Pharmaceutical Society's Laboratories, on the basis of the provisional standard solution supplied to them. The standard solution now recommended for general use cannot be assumed to be exactly equivalent to this, though comparative tests have not revealed a measurable difference. Apart from such difference as may exist, the unit now proposed will, therefore, correspond to one of those already current. It is of convenient size, as representing the order of dose required for daily administration to a rat to produce healing of rickets in a curative test; that is to say, the daily dose suitable for the conditions of a particular test will be neither a large multiple nor a small fraction of the unit proposed.

The Committee mention three biological methods of estimating antirachitic effect, which they are able to recommend. Their recommendations on this matter are obviously not meant to be exclusive. They have mentioned the methods in which, from experience of their own investigations, they have confidence; but their statement is not intended to preclude the use of other biological methods that already exist, or of better ones that further investigation may produce, provided that in every case the test is based on a comparison with the fixed standard.

As indicated in another of the Committee's recommendations, the quantity of the standard solution available will not allow it to be circulated in large quantities. Its distribution will, of necessity, be limited to those institutions which have the staff and equipment needed for its accurate application in biological assay, including proper facilities for its preservation without loss of activity. Unless the number of institutions requiring it proves to be much greater than that to which other standards are distributed, it should be possible to supply samples once or twice a year to each regular recipient for some years to come, before the preparation of a new batch of the standard solution will be required. It is proposed to make the first issue about the end of September of this year, and a memorandum giving details for the use of the standard will be sent with each first sample. It will greatly facilitate the completion of arrangements if applications from institutions wishing to receive the standard are made as early as possible, so that the Department may be able to estimate the number of separate samples likely to be required for the first and subsequent issues. All such applications should be made to:—

The Director of the Department of
Biological Standards,
National Institute for Medical Research,
Hampstead, London, N.W.3.

Methods of Estimation

I. Animals for Use in Estimations.—As vitamin D can be stored in the animal body, it is essential that the reserves of the factor in the young rats to be used for tests should be so small that a measurable degree of rickets may develop in a reasonable time when the rats are given a rachitogenic diet. The diet of the stock rats

must therefore be strictly controlled. One diet which has been found to be satisfactory for several generations of rats consists of the following:—

Yellow maize (ground)	65%
Whole wheat (ground)	20%
Caseinogen	9%
Dried yeast (brewers')	5%
Sodium chloride	0.5%
Calcium carbonate	0.5%

This mixture is given in the dry state to the rats ad lib. In addition, porridge with milk is given every day, about 2 grams of liver or beef twice a week and fresh green stuff, lettuce or watercress, about 2 grams twice a week. Twenty per cent of dried whole milk may be added to the dry mixture and the porridge omitted. It is desirable to buy the yellow maize and wheat whole and grind them in the laboratory to ensure the presence of the germ in the mixture. The casein need not be pure. It should be of good quality. The light white casein made by the British Drug Houses has proved satisfactory for this purpose. Obviously this diet is not of uniform quality throughout the year, but it is one which has been found to serve its purpose through some two and a half years in one laboratory. (Coward Cambden and Lee, *Biochem. J.*, 1932, **26**, [in the press].)

The room in which the colony of rats is housed should face north so that no direct sunlight may fall on the rats through open windows.

II. Tests (a) The "Line" Test.—Young rats of 50–60 grams weight are given a rachitogenic diet for 3–4 weeks during which time they develop a severe degree of rickets provided their original reserves of vitamin D are low, and provided also that they have not been exposed to strong sunlight (even through window glass) during this time. A simple and well-tested diet suggested by Steenbock and Black (*J. Biol. Chem.*, 1925, **64**, 263) is used in many laboratories. It consists of:—

Yellow maize (ground)	76%
Gluten flour	20%
Sodium chloride	1%
Calcium carbonate	3%

It owes its rachitogenic property to its high-calcium, low-phosphorus content, (Ca:P = 4:1), as well as to its low content of vitamin D.

During this preparatory period the rats of any one litter may be kept in a single cage and bedded on sawdust if it is known that the sawdust has not been exposed to direct sunlight or other source of ultraviolet rays. Otherwise screens of wire mesh should be used.

The preparatory period is followed by a test period of 10 days, during which the rats are kept in separate cages and given doses of the standard preparation of vitamin D or of the substance under examination. At the end of the test period, the animals are killed, the distal ends of the ulnae and radii are removed and (each pair with the rat's number attached) are immersed in 4% formaldehyde for about 24 hours. This clears them somewhat and makes the subsequent pictures easier to read. The ulnae and radii are then separated with a cut by a sharp scalpel, and each bone is cut into two longitudinally. The halves are then placed, cut surface uppermost in a small dish of 1.5% silver nitrate for a few minutes and exposed to light. This converts the calcium phosphate into silver phosphate which is reduced by light to black colloidal silver. Thus the calcified parts of the bone are seen at once as blackened areas in the sections and the degree of rickets and of healing can be determined from the blackened areas in these cut surfaces (McCollum, Simmonds, Shipley & Park, *J. Biol. Chem.*, 1922, **51**, 41). Microscopic examination is superfluous.

The clearing in formaldehyde may be omitted, but if it is, blood may be left in the metaphyses (especially of rats with very severe rickets) which on a superficial examination may be mistaken for blackened bone.

The degree of rickets to be aimed at is one in which the depth of the uncalcified metaphysis is approximately equal to its width. If this degree is attained, healing will begin as a thin plate of calcification in this region, which will appear as a narrow line in the area of the section. Greater healing will show as a wider line and complete healing as almost complete calcification of this part, only a very narrow layer of cartilage remaining between the epiphysis and the end of the shaft of the bone. If a less severe degree of rickets is attained, no "line" appears in the section and the test must be considered as a prophylactic one; that is, the potency of a dose must be judged by the degree of rickets produced as indicated by the width of the metaphysis; the greater this width, the more

severe is the degree of rickets produced and therefore the less potent is the test dose.

Comparisons between test substance and standard must always be made on rats of the same litter, for even with the greatest care in the breeding colony, the variations in reserves of vitamin D are greater between different litters than between members of any one litter. A convenient dose of standard is a half or one unit. The dose of the substance to be tested is determined by a consideration of the possible potency of the substance. A sample of cod-liver oil might be expected to contain 50–200 units of vitamin D per gram, and therefore a suitable arrangement of test doses for a litter of seven rats would be:—

(a) No dose
(b) 0.005 grm. C.L.O. daily
(c) 0.01 grm. C.L.O. daily
(d) 0.02 grm. C.L.O. daily
(e) 0.25 unit vitamin D daily
(f) 0.5 unit vitamin D daily
(g) 1.0 unit vitamin D daily

Corresponding degrees of healing given by one or more doses of cod-liver oil and one or more doses of vitamin D would indicate the potency of the oil which would be expressed as a number of units per gram. Results of such a test are, however, frequently not so regular as to justify a conclusion from only one litter of rats. It is desirable, if time permits, to make one test on the plan just described, then to take another litter of rats prepared similarly and give several of the rats the same dose of cod liver oil and several others the dose of vitamin D which seemed from the first test to correspond with it. If these give approximately equal degrees of healing, the first estimation is confirmed; if not, then the difficulty is to know how much more activity is indicated by the greater amount of healing. The ideal procedure would be to use further litters of rats, giving half of the rats of each litter a fresh dose of test substance and the other half of each litter the same dose of standard, in the hope of finding, eventually, the dose of test substance which would have an effect equal to that of the dose of vitamin D. This would involve some system of averaging results and the simple method of drawing up an ideal series of healing bones and giving numerical values to the different members of the series suggests itself. By comparison

with this series, values may be assigned to bones of test animals and results averaged.

A great economy of time could, however, be effected if the relation between the average degree of healing and the dose of vitamin D given could be determined in every laboratory by an experiment definitely planned for that purpose; for, having obtained a certain difference in healing on a given dose of standard and a given dose of test substance, that difference could be assigned to a certain ratio of the vitamin D content of the two doses, and the vitamin D content then calculated from the dose of vitamin D (standard) actually given. A method based on this principle has been worked out by F. J. Dyer (*Quart. J.* Pharmacol) and by Key and Morgan (*Biochem. J.*, 1932, **26**, 196).

(b) Chemical Analysis of Bones.—The ash content of the bones of a rat is modified by the amount of vitamin D in the diet, particularly if the diet is strongly rachitogenic as is the Steenbock and Black diet already described. This fact is made the basis of a test for vitamin D (Chick, Korenchevsky and Roscoe, *Biochem. J.*, 1926, **20**, 622).

Young rats weighing 40–50 grm. are given a rachitogenic diet together with a daily dose of the substance under test, or the standard preparation of vitamin D for a period of 4–5 weeks. The doses should be arranged amongst the rats of each of six litters in the manner suggested for the line test but doses about one-tenth of those doses are sufficient for this test as it is a prophylactic one. If there is no knowledge of the probable potency of the substance, doses varying much more than 100% must be used as a trial. When some idea of the potency has been obtained, more accurate information can be gained by a second test on more closely related doses. Thus, there should be about 6 rats on each dose of the test substance and of the standard. At the end of the 28 or 35 days, the rats are killed, the femora and humeri are removed and cleaned as carefully as possible from tissue and other debris with muslin or cheese cloth, weighed, dried to constant weight at 105° C., weighed again, extracted in a Soxhlet with alcohol for 24 hours to remove fatty compounds, dried, weighed again, and ashed in weighed crucibles by heating first over a Bunsen flame, then in a muffle furnace and weighed again. The ash is expressed as a percentage of the dry, fat-extracted bone.

In this method, as in the last, great variations are encountered, but it has been found that, in general, the use of six rats in each dose

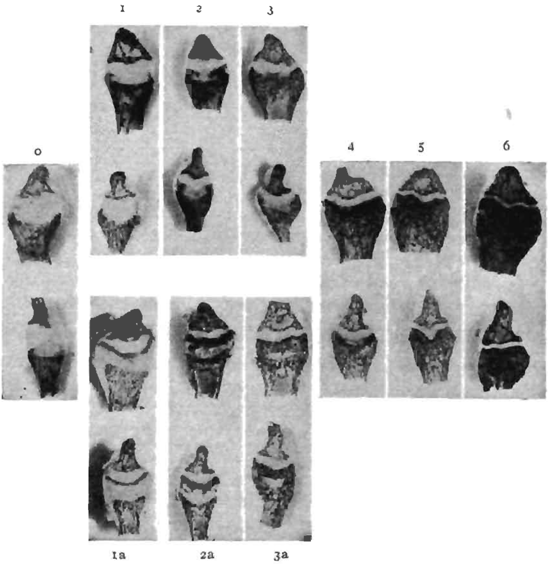

FIG. 6.—Healing of rachitic bones. Ulnae and radii of 10 rats in which a severe degree of rickets had been developed by feeding on the Steenbock rachitogenic diet for 3 weeks. Different degrees of healing were produced by giving different doses of vitamin D for 10 days.

Healing of rickets may proceed as in the series 0, 1, 2, 3, 4, 5, 6 in which the narrowing of the metaphysis is the criterion of healing, or as in the series 0, 1a, 2a, 3a, 4, 5, 6, in which the width of the "line" of healing and subsequent narrowing of the metaphysis are the criteria of healing.

(one from each litter on each dose) gives averages of ash content graded to the dose of vitamin.

A laboratory could determine for itself what differences in ash content were produced by differences in doses of vitamin D as described for the "Line test" and thus evaluate differences between

test substances and standard found in later experiments, but, obviously, conditions would have to be kept uniform; the basal diet of the experimental animals must always be the same, the same room and lighting must be used and the same treatment of the stock colony must be continued. It would be impossible to assume that a certain difference between two doses of vitamin D would bring about equal differences in ash content in different laboratories. Until a laboratory has established its own relation between ash content to dose of vitamin given, the only procedure is to test several different doses of the unknown against several doses of standard, as outlined above (Hume, Pickersgill and Gaffikin, *Biochem. J.*, 1932, **26**, 488).

(c) **The X-ray Method.**—This may be carried out either as a curative test like the "Line test," or as a prophylactic test like the chemical test. The arrangement of the animals of this test is the same but the estimation of the degree of rickets produced and of the subsequent healing may be estimated without the killing of the rat. The X-ray photograph of the bone gives essentially the same picture as the line test. Thus, in a curative test, the degree of rickets can be determined before the dosing of unknown or of standard is begun. The healing brought about by the dosing is similarly recorded. By constructing a scale such as the one suggested for the line test, it is possible to relate differences in degrees of healing with differences in doses of vitamin D. Without this, of course, full comparisons between several different doses of unknown and several different doses of standard must be made (Bourdillon, Bruce, Fischmann and Webster, *Med. Res. Council, Special Report Series*, No. 158, [1932]).

ESTIMATION OF VITAMIN E

The examination of foodstuffs for vitamin E has not yet assumed practical importance, and outside the United States comparatively little experimental work on the subject has been carried out.

Method of Evans (*University of California Monograph,* No. 8, 1927).—Young doe rats of about 50 grams weight are selected from a healthy colony and fed on a diet consisting of:—

Casein	18%	Lard	15%
Corn starch boiled with water, dried, and ground	54%	Butter fat	9%
		Salts	4%

In addition each rat is given 0.4–0.6 grm. of dried yeast daily.

As soon as the vagina of each rat opens, daily examinations of the vaginal contents are made. When the rat is about 10 weeks old (this is a black-and-white strain which matures somewhat earlier than the pure-bred albino strain) it is mated with a buck of proved fertility. If sperm are found in the vaginal smear at the next oestrous cycle, no more smears are examined for 14 days. The next smear taken will consist of a mass of red blood cells or small clot of blood, quite unmistakable by the naked eye. This will show that fertilisation has taken place. Seven days after this, under normal circumstances a litter of living young would be born. It may be born, even under the conditions described for the feeding of these animals, if the doe has been richly provided with reserves of vitamin E from her parent, otherwise no litter will be born. The increase in weight noted on the 14th day of pregnancy may be continued for a day or two more, then a gradual loss of weight occurs during some 10 days. The foetuses are resorbed.

Does which have had one resorption are considered ready for testing substances for vitamin E. The same basal diet is continued, vaginal smears are examined daily, mating is made possible at the occurrence of a cycle and a dose of test substance is given on the day when sperm has been found in the vagina, that is, the day after a positive mating has taken place. Apparently it is immaterial whether the whole dose of vitamin E is given on the day after mating or on any day up to the fifth day after mating. For a substance of low vitamin E content, the dose may have to be a daily one throughout gestation and should then be begun not later than the fifth day after copulation.

The birth of living young 21 days after copulation is considered proof of the presence of vitamin E in the substance tested.

To make this test quantitative, the lowest dose of the test substance which will produce the birth of living young under the conditions described must be determined. Comparisons with wheat germ may be made. 250 mg. of wheat germ daily during gestation has been found to be a minimum dose. 25 mg. of oil extracted from wheat germ has also been found to be a minimum dose. It is at least probable that different samples of wheat germ and of wheat germ oil have different vitamin E potencies. These figures, therefore, should be taken as approximate only.

There is no standard preparation of vitamin E.

ANNEX IV

PREFACE

A most important advance in the estimation of vitamins was made in June, 1931, when a conference was held in London, under the auspices of the Health Organisation of the League of Nations, to consider the question of standards of reference for the estimation of the vitamins. International agreement was reached on a number of important points, and the way was prepared for the adoption of standards for 4 vitamins. The writers are greatly indebted to the Health Organisation of the League of Nations for permission to reproduce this report in full.

C.H.1055(1).

REPORT OF THE CONFERENCE ON VITAMIN STANDARDS, HELD AT LONDON FROM JUNE 17TH TO 20TH, 1931

LIST OF PARTICIPANTS

Chairman:

Professor E. MELLANBY, Professor of Pharmacology, University of Sheffield, Sheffield.

Participants:

Professor J. C. DRUMMOND, Professor of Biochemistry, University College, University of London.

Professor H. VON EULER, Professor of Biochemistry, University of Stockholm, Stockholm.

Professor L. S. FRIDERICIA, Director, Institute of Hygiene of the University, Blegdamsvej 21, Copenhagen.

Professor B. C. P. JANSEN, Professor of Physiological Chemistry, University of Amsterdam, Amsterdam.

Professor E. V. MCCOLLUM, Professor of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, Baltimore.

Professor E. POULSSON, Director of the State Vitamin Institute, Skoyen-Oslo.

Mme. G. L. RANDOIN, "Directeur du Laboratoire de physiologie au centre de recherches sur l'alimentation (Institut de recherches agronomiques), Directeur du laboratoire de physiologie de la nutrition à l'Ecole des Hautes-Etudes," Paris.

Professor A. C. SCHEUNERT, "Direktor des Tierphysiologischen Instituts der Universität," Leipzig.

Dr. A. SEIDELL, Chemist, National Institute of Health, United States Public Health Service, Washington, D.C.

Professor H. STEENBOCK, Professor of Agricultural Chemistry, University of Wisconsin, Madison, U.S.A.

Professor A. WINDAUS, "Direktor, Allgemeines Chemisches Laboratorium der Universität, Göttingen," Germany.

Technical Secretaries:

Dr. H. CHICK, Lister Institute for Preventive Medicine, London.

Dr. W. R. AYKROYD, Lister Institute for Preventive Medicine, London.

The following were also present:

Dr. C. E. BILLS, Director of Research, Mead Johnson and Co., Evansville, Indiana, U.S.A.

Dr. R. BOURDILLON, National Institute for Medical Research, London.

Dr. KATHARINE COWARD, Pharmaceutical Society's Laboratories, London.

Professor R. A. PETERS, School of Biochemistry, Oxford.

Dr. O. ROSENHEIM, National Institute for Medical Research, London.

Dr. S. S. ZILVA, Lister Institute for Preventive Medicine, London.

In the following recommendations, the question of standards and units for the four following vitamins only is considered:

I. The fat-soluble Vitamin A.
II. The antirachitic Vitamin D.
III. The antineuritic Vitamin B (also known as Vitamin B_1).
IV. The antiscorbutic Vitamin C.

It is the general opinion that, in the present state of our knowledge, only these vitamins can be profitably discussed in this connection.

APPENDIX

I. The Fat Soluble Vitamin A

(a) *International Standard.*

The Conference recommends that carotene be accepted as an international provisional standard of reference for Vitamin A and that a selected sample of cod-liver oil be held in view as a possible secondary standard.

(b) *Mode of Preparation.*

It was decided that the information available does not yet justify the selection of one isomer of carotene as a standard. The similar biological activity of the two isomers that have recently been described is further justification for adopting as the provisional international standard a mixture prepared in an approved manner.

It was decided to employ a preparation of carotene made from carrots by Willstätter's method and purified by recrystallisation by the method described in the memorandum issued by the Department of Biological Standards, National Institute for Medical Research, London, until the melting-point determined is above 179°. C. It was suggested that preparations should be made in various countries and despatched immediately and with all necessary precautions against decomposition to the National Institute for Medical Research, London, where they will be mixed to form a uniform preparation by the most suitable method. The details of this final purification are to be left to the discretion of the authorities of the National Institute. It was suggested that original preparations of, say, 4 to 5 grammes might be made in the following institutions:

Department of Physiological Chemistry, University of Amsterdam.

"Laboratoire de physiologie de la nutrition, Ecole des Hautes Etudes," Paris.

"Tierphysiologisches Institut," Leipzig.

National Institute for Medical Research, Hampstead, London.

Department of Agricultural Chemistry, University of Wisconsin, U.S.A.

Biochemical Department, University of Stockholm, and "Institut für Organische Chemie der Universität," Zürich.

School of Hygiene and Public Health, Johns Hopkins University, Baltimore, U.S.A.

(c) *Place of Preparation.*

It was decided that the National Institute for Medical Research, London, acting for this purpose as the *central laboratory on behalf of the Health Organisation of the League of Nations*, should be asked to undertake the final preparation of the sample of carotene to be used as the international standard for Vitamin A.

(d) *Mode of Distribution.*

It was considered desirable that, as far as possible, the standard preparation should be distributed to workers through the appropriate official institution in each country, preferably that now responsible for the distribution of similar biological standards. The material should be sent out in tubes of 10 mg.

(e) *Definition of Unit.*

The unit of Vitamin A recommended for adoption is the Vitamin A activity of 1 γ (0.001 mg.) of the international standard.

Note.—Daily doses of about 3 γ to 5 γ of the international standard, when administered to young rats suitably prepared on a Vitamin A-deficient diet, have been found adequate to restore growth and to cure xerophthalmia.

(f) *Permanence of the Standard.*

The Conference recommends that this international standard and unit be accepted provisionally for two years.

(g) *Subjects recommended for Future Investigations.*

It is highly desirable that, during the provisional period, further investigations of the standard should be made regarding the stability of the carotene preparation both when sealed in the original tubes and after it has been removed and dissolved for biological testing. In the latter connection, emphasis is laid on the importance of minimising contact with air or oxygen of the solutions used for

animal feeding. It is recommended that they be always stored in an inert gas and at low temperature.

It will be of great value if investigators submit to the League of Nations Health Organisation observations on the stability of the preparation, its behaviour in various solvents and under different conditions of storage, and any other information bearing on its use as a biological standard. The use of suitable "antioxidants" ("*antioxygènes*" in French) should, in particular, be studied.

(h) *Biological Methods for Estimation of Vitamin A.*

It was decided not to recommend any one particular method of conducting the biological assay, but to invite members of the Conference to submit to the League of Nations Health Organisation their observations on the value of the methods they have been using.

It is recommended that further attention be given to the methods based on the curative action of carotene for xerophthalmia and other lesions characteristic of Vitamin A deficiency, as well as to those based on increase in weight.

(i) *Selected Sample of Cod-Liver Oil for use as a possible Secondary Standard.*

It is recommended that a supply of an approved sample of cod-liver oil be obtained with the object of making a series of comparative tests to determine its suitability as an alternative standard. The Conference is informed that the United States Department of Agriculture is making arrangements for such a standard substance to be available in the United States during the coming year.

It was decided to ask the United States Department of Agriculture to obtain sufficient supplies of the oil for distribution in order to enable investigators of other countries to assay this oil in terms of the international unit of standard carotene. It is hoped thus to obtain evidence regarding the stability of Vitamin A in cod-liver oil as affected by conditions and time of storage.

II. The Antirachitic Vitamin D

(a) *International Standard to be adopted and Arrangements for Control.*

The Conference recommends that the standard solution of irradiated ergosterol at present issued from the National Institute of Medical

Research, London, be adopted as international Vitamin D standard for the next two years.

If within this period it should become necessary, owing to threatened exhaustion of the present supply, to replace this solution by a fresh standard, the equivalence shall be determined by experts of different countries who have had the opportunity of comparing the proposed new standard with the one at present issued. It is suggested that the following Institutions, among others, be invited to co-operate in those tests:

"Allgemeines Chemisches Laboratorium," Göttingen.

"Tierphysiologisches Institut," Leipzig.

Food and Drugs Administration Laboratory, Department of Agriculture, Washington, D.C.

Biochemical Department, University of Stockholm.

Department of Agricultural Chemistry, University of Wisconsin.

School of Hygiene, Johns Hopkins University, Baltimore.

"Laboratoire de physiologie de la nutrition, Ecole des Hautes Etudes," Paris.

Pharmaceutical Society, London.

(b) *Method of Preparation.*

1. It is recommended that, in the preparation of the solutions of irradiated ergosterol, used as standards of reference for Vitamin D (or as sub-standards), irradiation with ultra-violet light shall be done in ethereal solution in the absence of any appreciable traces of oxygen, and the solution should be kept in rapid motion. The conditions of exposure should be such as to transform between 30% and 80% of the ergosterol. The solution of the product and of further dilutions shall be made in a stable unsaturated natural vegetable oil, which has given a negative test for Vitamin D.

2. The standard solution of irradiated ergosterol at present issued from the National Institute for Medical Research, London (Standard Solution III), was, however, prepared as follows, in January 1929:

A 0.1% solution of the ergosterol in absolute alcohol was exposed for half an hour in a silica cell, 1 cm. thick, to the unfiltered radiation from a K.B.B. (Kelvin, Bottomley and Baird) mercury vapour lamp, taking 2.5 amperes and 125 volts at atmospheric pressure, at 15 cm. distance from cell to lamp. The resulting solution was mixed with a little olive oil, and then

evaporated at 45° C. at a low pressure to remove the alcohol. The concentrated oily solution thus obtained was diluted with pure olive oil to give a concentration corresponding to 1 mg. of the original ergosterol in 10 c.c. of olive oil at 18° C. The olive oil used was tested for stability and gave a negative test for Vitamin D.

The additional larger quantity of standard (prepared January 1931), which is available for distribution later if required, was prepared with observance of the general conditions indicated under 1.

(c) *Mode of Distribution.*

The National Institute for Medical Research, London, *acting for this purpose as the central laboratory on behalf of the League of Nations Health Organisation*, shall distribute to each country wishing to use the standard a sufficient quantity of the solution to enable the standard to be effectively applied according to the conditions in the particular country. Such quantity should be supplied only to a central institution nominated for the purpose by the country concerned, which will be responsible for the distribution either of the portion of international standard solution received or, wherever possible, of an equivalent sub-standard prepared by comparison therewith.

(d) *Stability of the International Standard.*

The stability of the standard solution at present issued from the National Institute for Medical Research, London, has proved satisfactory on the results of tests over a period of two years, when preserved at or below 0° C. with exclusion of air.

(e) *Definition of Unit.*

The unit of Vitamin D recommended for adoption is defined as the Vitamin D activity of 1 mg. of the international standard solution of irradiated ergosterol.

Note.—The international standard solution has been prepared to have such potency that approximately 1 mg. thereof given daily to a rachitic rat for eight successive days will produce a wide line of calcium deposits in the metaphysis of the proximal ends of the tibiæ and of the distal ends of the radii.

(f) *Permanence of the Standard.*

The international standard at present recommended shall be regarded as provisional for the next two years, in the hope that a

more stable crystalline substance may in the meantime become available.

(g) *Subjects recommended for Further Investigation.*

1. The influence of various oils as solvents upon the stability of solutions of irradiated ergosterol.

2. Further investigation of the crystalline antirachitic substances recently isolated from irradiated ergosterol. It was decided that Professor Windaus and Dr. Bourdillon shall be asked to investigate the constancy of the physical properties of the crystalline products recently isolated by them respectively, in order to determine whether these may be regarded as pure substances. If so, the potency of these products should be accurately compared with that of the (present) international standard at intervals of three months during the next two years, in order to compare the stability in each case. If the results are satisfactory, it is hoped that one of these crystalline substances may eventually replace the solution of irradiated ergosterol as international Vitamin D standard.

3. The toxicity of the present standard and of the crystalline products should be investigated (see also Note at the end of this appendix).

(h) *Biological Methods for Estimation of Vitamin D.*

In using the international standard solution for the determination of the antirachitic potency of unknown preparations, it is recommended that not fewer than twenty rats (preferably more) be used for a determination, half of these to receive the standard and the remaining litter-mates the unknown substance. Provided this precaution is observed, it is considered permissible to use various biological methods of estimation, either prophylactic or therapeutic. For instance, the "line" test, X-ray examination or determination of the bone ash, are all considered reliable methods.

III. The Antineuritic Vitamin B

(a) *International Standard.*

The Conference recommends the adoption, as international standard, of the adsorption product of the antineuritic Vitamin B prepared in the Medical Laboratory, Batavia (Java), by the method of Seidell, as described by Jansen and Donath.

(b) *Terminology.*

The international standard preparation should be known as the "standard adsorption product of the antineuritic Vitamin B."

(c) *Method of Preparation.*

The international standard is prepared by extracting rice polishings with water, sufficient sulphuric acid being added to make the *p*H 4.5. Salicylic acid to a concentration of 0.2 % and toluene are then added to prevent bacterial decomposition. The process of extraction is continued for two days, after which the solution is filtered. For each 100 kilogrammes of the original rice polishings, 3 kilogrammes of fuller's earth (specially selected for its adsorptive powers) are added to the solution, which is then stirred for twenty-four hours. Subsequently, the solution is filtered off and the fuller's earth, after being washed with water and alcohol, is dried; 3 kilogrammes of the fuller's earth adsorbate represents the antineuritic Vitamin B from 100 kilogrammes of rice polishings.

(d) *Place of Preparation.*

It is recommended that the Medical Laboratory, Batavia, Java, should be asked, through Professor Jansen, of Amsterdam, to prepare a batch of 25 kilogrammes of the standard preparation. This should provide an adequate supply for many years.

(e) *Place of Distribution.*

It is suggested that this batch of standard adsorption product of antineuritic Vitamin B should be kept at the National Institute for Medical Research, London, *acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations.*

One hundred grammes would be an amount suitable for distribution to individual laboratories. No special precautions are necessary in keeping this preparation, except that it should be stored in a dry place. In the presence of moisture, bacterial decomposition readily takes place.

(f) *Definition of Unit.*

The unit recommended for adoption is the antineuritic activity of 10 milligrammes of the international standard adsorption product.

Note.—A daily dose of 10 to 20 mg. of this preparation is required to maintain normal growth in a young rat on a diet deficient in the antineuritic Vitamin B but complete in all other respects, including the antidermatitis Vitamin (B_2); the curative "day dose" for a pigeon (300 grammes weight) suffering from polyneuritis on a diet of polished rice is about 20 to 30 mg. (method of Kinnersley and Peters).

(g) *Permanence of the International Standard Recommended.*

This standard adsorption product should serve as a provisional international standard for five years, or until advances in the knowledge of this vitamin make a revision desirable.

(h) *Recommendations for Further Investigations.*

1. The standard adsorption product should be investigated for its content of other vitamins B.

2. Although there is no evidence that loss of potency is liable to occur in the standard adsorption product, the Conference suggests that the following laboratories be asked to undertake a further investigation of its stability:

Department of Physiological Chemistry, University of Amsterdam.
National Institute of Health, United States Public Health Service, Washington, D.C.
Institute of Hygiene, University of Copenhagen.
School of Biochemistry, University of Oxford.
"Tierphysiologisches Institut," Leipzig.
Biochemical Institute, University of Stockholm.
"Laboratoire de physiologie au Centre de recherches sur l'alimentation (Institut des recherches agronomiques)," Paris.
Lister Institute for Preventive Medicine, London.

(i) *Biological Methods for Estimation of the Antineuritic Vitamin B.*

The Conference expresses no opinion on the relative merits of current biological methods for estimation of the antineuritic Vitamin B (as recorded in the report on this vitamin, presented to this Conference and in the literature generally). It considers that good evidence is provided by that report that the different methods described, either prophylactic or curative in type, and employing

either the rat or the pigeon as experimental animal, may yield equally valid results.

IV. The Antiscorbutic Vitamin C

(a) *International Standard.*

The Conference recommends the adoption as international standard of the fresh juice of the lemon, Citrus limonum.

(b) *Definition of Unit.*

The unit of the antiscorbutic Vitamin C recommended for adoption is the Vitamin C activity of 0.1 c.c. of fresh juice of the lemon, *Citrus limonum.*

Note.—This is about 1/10th of the daily dose necessary to prevent development of macroscopic scorbutic lesions in a young guinea-pig maintained on a scurvy-producing diet.

(c) *Method of Use.*

The fresh lemon juice used as standard may be decitrated as follows: to the expressed juice after filtration through muslin an excess of calcium carbonate is added until effervescence stops. After standing for one hour, the mixture is filtered through a Buchner funnel. The decitrated juice should have a reaction of *p*H about 6 and should be administered to the experimental animal within two hours after filtration.

(d) *Permanence of Standard.*

This international standard shall be regarded as provisional for the next two years.

Note on the Toxicity of Irradiated Ergosterol

In view of the toxic effects which have been reported after administration of certain specimens of irradiated ergosterol, this Conference suggests the advisability of testing all preparations of irradiated ergosterol, destined for medicinal use, for toxicity as well as for antirachitic potency.

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THE HORMONES

BY K. CULHANE, B.Sc., A.I.C. AND S. W. F. UNDERHILL, M.A., B.M., B.Ch.

Introduction.—The hormones are the active principles of the glands of internal secretion. The glands of vertebrate animals may be divided into two groups; the first or glands with an external secretion pour the secretions of their cells into a duct by which the fluids are passed to an internal or external surface. The second group or glands with an internal secretion have no ducts and discharge their secretions directly into the blood or lymph stream.

The active principles of the latter supply a continuous stimulus of varying intensity to a number of different organs and tissues, whereby many of their functions are correlated; the response to such stimulation is relatively slow. This correlation may be contrasted with that depending upon the nervous system; in this case the stimulation is usually brief and the effect immediate.

The hormones are used clinically both to replace the natural secretion when that is deficient, and also as drugs to modify the functions of the body in some desired manner. Although active extracts have been obtained from the majority of glands possessing an internal secretion, in only three or four cases have pure crystalline compounds been isolated, and in only two has the isolation been followed by determination of the chemical constitution, namely, in the case of adrenaline and thyroxine.

Owing to their high activity and their important position in modern therapeutics, it is essential that they should be submitted to adequate tests before administration. In the case of the majority only physiological methods are available for the identification and estimation of the active principles and even when the hormone has been obtained in crystalline condition physiological tests are generally necessary and always desirable.

THYROXINE AND THE THYROID GLAND

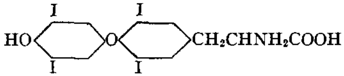
The thyroid gland lies in the neck on either side of the larynx and trachea. It consists of two lobes frequently joined across the front of the upper part of the trachea by an isthmus. It is present in all vertebrates and is developed as an out-growth from the entoderm of the floor of the pharynx, from which in most species it subsequently becomes detached. In amphioxus and ammocoetes, however, the connection with the pharynx remains open. It is composed of small closed vesicles of varying shape, usually irregularly spheroidal and lined with a columnar or cubical epithelium; the vesicles are filled with colloid, a viscid yellowish-red material. The cells secrete thyroxine, probably in the form of a peptide containing the laevo-rotatory isomer in combination with a protein molecule. Thyroxine is one of the hormones regulating the normal growth of the cells and tissues of the body, and it also has a general stimulating effect upon their metabolism.

THYROXINE

Thyroxine is obtained from the thyroid gland or can be prepared synthetically. When employed in therapeutics it is commonly administered in the form of dried thyroid gland, obtained from the sheep or other domestic animals used for food by man.

Constitution

Thyroxine ($C_{15}H_{11}O_4NI_4$) is a tetraiodo derivative of the *p*-hydroxy-phenyl ether of tyrosine.



It is the only natural product so far known which is a derivative of diphenyl oxide.

The constitution was suggested by the following observations and proved beyond all doubt by its synthesis (C. R. Harington, *Biochem. J.*, 1926, **20**, 293; Harington and G. Barger, *Ibid.*, 1927, **21**, 169).

1. The nitrogen is all present in amino form, as shown by the action of nitrous acid.

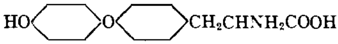
2. There is one acid group and one hydroxyl group.

3. Fusion with potassium hydroxide at 310° in an atmosphere of hydrogen gives *p*-hydroxybenzoic acid, quinol, ammonia, and oxalic acid.

4. Exhaustive methylation of the reduced product, desiodothyroxine, results ultimately in a substance which loses trimethylamine when boiled with alkali, giving a salt of an unsaturated acid.

5. The unsaturated acid oxidised with permanganate gives oxalic acid and an aldehyde, which upon further oxidation gives an acid, $C_{14}H_{12}O_4$, stable to alkaline permanganate.

Desiodothyroxine or thyronine, both by its degradation and by its synthesis, was proved to have the composition represented by the formula



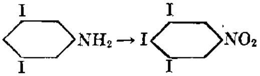
Finally the position of the four iodine atoms was proved by the synthesis of thyroxine itself by a method which has now become a commercial process.

Preparation

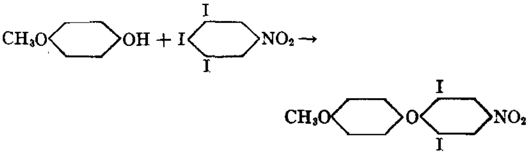
1. SYNTHESIS

The synthetic preparation may be summarised by the following series of reactions:—

1. *Diiodo p-nitraniline in solution in sulphuric acid is converted* by treatment with sodium nitrite followed by potassium iodide to 3:4:5- triiodonitrobenzene

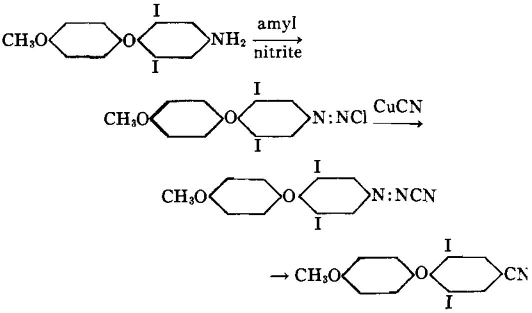


2. Triiodonitrobenzene is coupled with quinol monomethyl ether by boiling with dry potassium carbonate in methyl ethyl ketone



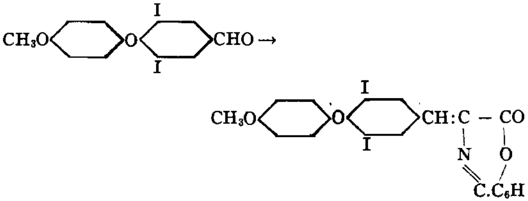
3. 3 : 5-Diiodo-4(4′methoxy-phenoxy)nitrobenzene is reduced by means of stannous chloride to the corresponding amino compound.

4. The amino compound is converted to the benzonitrile by Sandmeyer's reaction:—

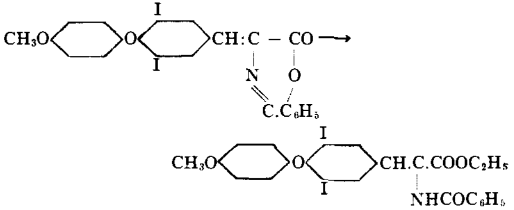


5. The nitrile is converted to the corresponding aldehyde by treatment with stannous chloride.

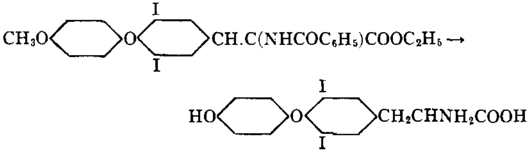
6. The az-lactone is formed by treatment of the aldehyde with hippuric acid



7. α-Benzoylamino 3 : 5-diiodo-4(4′methoxy phenoxy) cinnamic ester is formed by treatment of the az-lactone with alcohol in sulphuric acid



8. Reduction of the cinnamic ester with hydriodic acid and red phosphorus gives β-(3:5-diiodo-4(4'hydroxyphenoxy) phenyl) α-amino propionic acid.



9. Finally the last two atoms of iodine are introduced by treatment in solution in concentrated ammonia with iodine in potassium iodide. The ammonium salt of thyroxine is thus obtained. It is dissolved in alcohol containing a little sodium hydroxide and the solution filtered; the filtrate is boiled and acidified with acetic acid. A colourless crystalline precipitate separates in rosettes and sheaves of colourless needles. A further amount can be obtained from the mother liquors.

2. EXTRACTION

Harington's method of extraction from the glands results in a higher yield than that obtained by the older method of Kendall which gave only 0.0011% of the fresh weight. Harington's method is as follows (*Biochem. J.* 1926, **20**, 296):—

500 grm. of desiccated glands are boiled for 5–6 hours under a reflux condenser with 5,000 c.c. of a 10% solution of crystalline barium hydroxide. The mixture is allowed to stand overnight and

the heavy dark precipitate filtered off through Buchner funnels. This is set aside, and the clear yellow filtrate is acidified with hydrochloric acid until nearly acid to congo red. A light-coloured precipitate is formed and allowed to settle for some hours, when it is separated by decantation and filtration. The precipitate is washed with water, the washings and filtrate being rejected. The precipitate is dissolved in 200 c.c. of water containing ammonia, and crystalline barium hydroxide is added to the extent of 40%. The excess of ammonia is boiled off and the solution is heated for 18 hours under a reflux condenser, the temperature being kept below 100°. It is filtered hot through a Buchner funnel, and the precipitate is washed with a little hot water. The dry precipitate is ground up and suspended in 250 c.c. of 1% sodium hydroxide solution, the suspension is boiled, and a strong solution of sodium sulphate is added in excess while the liquid is still boiling. The heating is continued for a few minutes, after which the barium sulphate is filtered off. 50% sulphuric acid is added to the boiling filtrate until it is just acid to congo red, when a precipitate forms. On further boiling the character of the precipitate changes from light and flocculent to heavy and granular. The mixture is then cooled, filtered, and the precipitate is dissolved in about 20 c.c. of normal sodium hydroxide solution, alcohol is added to bring the strength to 80%, and a small amount of tar is filtered off. The filtrate is boiled and treated with 10 c.c. of 33% acetic acid, when partly crystalline thyroxine separates in a yield of about 0.08%.

The dark-coloured precipitate that was set aside at the beginning of the extraction is treated separately. It is ground up and boiled with 2,000 c.c. of 2% sodium hydroxide solution, a slight excess of sodium sulphate being added to the boiling solution. The precipitate is filtered off, the filtrate acidified with hydrochloric acid, and the resulting precipitate is treated in the same way as the light coloured precipitate formed above. This gives a further yield of about 0.045%, making a total of 0.125% (equivalent to about 0.027% of the fresh weight). The thyroxine is purified by repeated solution in alkaline alcohol and precipitation with acetic acid, but the last traces of coloured impurity must be removed as follows:—After one recrystallisation by the above method, it is dissolved in boiling 0.5% sodium carbonate solution (about 75 c.c. are needed for 1 grm.). A heavy white microcrystalline precipitate separates on

cooling, and this is centrifuged off after standing for some time in the ice chest. The precipitate is dissolved in 80–90% alcohol with a little sodium hydroxide, and the thyroxine is precipitated by addition of excess of 33% acetic acid to the boiling solution. A perfectly white product is obtained.

Resolution of dl-Thyroxine.—The resolution of thyroxine is a matter of some importance, because the activity of the laevo compound is definitely greater than that of the dextro or racemic substances. The isolation of the laevo compound from the glands has been effected by means of enzymes, but the yield is small (C. R. Harington and W. T. Salter, *Biochem. J.*, 1930, **24**, 456). Pure *d*- and *l*-thyroxine have been prepared by Harington by resolving *dl*-formyl diiodo thyronine and iodinating the products (*Ibid.*, 1928, **22**, 1429).

Properties

Thyroxine is a colourless, odourless substance which crystallises in rosettes and sheaves of fine needles. It darkens at 220° and melts (with decomposition and evolution of iodine) at 231–233° (heating at the rate of about 3° per minute). E. C. Kendall and A. E. Osterberg (*J. Biol. Chem.* 1919, **40**, 265) state that the crystalline form varies with the method of preparation. Thus, if it is separated from solution by sulphuric, hydrochloric or acetic acid, the crystals are in the form described above, but if it is precipitated from an alkaline solution with carbon dioxide, a finely divided precipitate consisting of fine curved hair-like crystals is obtained. This form is more sensitive to light and readily turns a yellowish brown. On precipitation from dilute alkali by ammonium chloride, another form is obtained of lower melting point, which is believed by Kendall and Osterberg to be hydrated.

Pure thyroxine is insoluble in water and all ordinary organic solvents. It is soluble in cold solutions of alkali hydroxides, provided the concentration of the alkali is not high. The insolubility of the compound is shown by the following figures given by Kendall:—

1 part is soluble in 84,000 parts of 0.6 *N* hydrochloric acid.

1 part is soluble in 77,000 parts of *N* sulphuric acid.

1 part in 263,000 remains in solution at 25° when dilute hydrochloric acid is added to a solution of the sodium salt.

1 part in 815,000 remains in solution at 25° when thyroxine is precipitated from water by carbon dioxide.

It is precipitated from solution in alkalies by addition of acid and it does not dissolve in excess. It dissolves in sodium carbonate solution only on boiling; in presence of alkali hydroxide or mineral acid it is soluble in 90% alcohol, from which, however, it is precipitated by acetic acid. It is soluble in formic acid.

On heating thyroxine with hydriodic acid at 140°, tyrosine is produced. Thyroxine is fairly easily reduced. Weakly alkaline solutions turn black in sunlight, the iodine being split off.

When nitrous acid is added to an alcoholic solution or to an aqueous suspension, in presence of a trace of hydrochloric acid, a yellow colour develops which turns pink on addition of ammonia. This is the best known chemical test for thyroxine, but it is also given by other ortho-iodo-hydroxyl derivatives of benzene.

d-Thyroxine m.p. 237° (with decomposition). 0.74 grm. dissolved in 6 grm. of 0.5 N sodium hydroxide and 14 grm. of alcohol, gave $[\alpha]_{5461}^{21^\circ} + 2.97^\circ$.

l-Thyroxine m.p. 235–236° (with decomposition). 0.66 grm. dissolved in 6.07 grm. of 0.5 N sodium hydroxide and 13.03 grm. of alcohol, gave $[\alpha]_{5461}^{21^\circ} - 3.2^\circ$.

The laevo form is from 2–3 times as active physiologically as the dextro form and occurs as such in the gland. All methods of extraction, except that by enzymes, cause racemisation, which partly explains why pure thyroxine is less active than preparations of the desiccated gland, of equal thyroxine content.

SALTS, DERIVATIVES, AND RELATED COMPOUNDS

Monosodium thyroxine is obtained from thyroxine by dissolving in 1% sodium hydroxide and precipitating by bubbling in carbon dioxide (100 mg. thyroxine in 100 c.c. sodium hydroxide). The suspension is heated until it dissolves; on cooling, crystals separate. The crystals must be washed on the filter with 10–15% sodium chloride solution, as about 40% dissolves in cold water and passes through the filter.

Monosodium thyroxine is soluble in 0.1% sodium carbonate solution to the extent of 1 part in 850 at 20°.

Monosodium thyroxine is official in the B. P. 1932 as thyroxin-sodium.

Disodium Thyroxine.—By using stronger sodium hydroxide the disodium salt separates. 100 mg. of thyroxine are dissolved in 20 c.c. of dilute sodium hydroxide solution, and 150 c.c. of 10% sodium hydroxide solution containing 20% sodium chloride added. The crystals are insoluble in 15% sodium chloride solution.

The potassium derivatives are prepared similarly. Many other metal salts have been obtained in crystalline form, but their properties are not described, beyond the fact that they are unstable and hydrolysed by boiling water.

Iodothyreo-globulin.—Thyroxine has been shown by Harington and co-workers to occur in the gland as iodothyreo-globulin, a compound which has been recognised for a number of years as an active constituent. It is extracted as follows:—Frozen thyroid glands are minced rapidly and suspended in one part by weight of 0.02% sodium hydroxide containing 1% sodium chloride. The suspension is shaken for 3 hours and then allowed to settle for 12 hours. It is strained through butter muslin, and the solid residue is re-extracted. The combined extracts are chilled and the fat is removed. The solution is then adjusted to *p*H 5.0 and sodium sulphate is added to the extent of 1%. Upon rapidly boiling the mixture by bubbling in steam, at the same time applying external heat, iodothyreo-globulin coagulates. It contains 97% of the total iodine of the extract. From this material, by the action of pepsin and trypsin, Harington and Salter (*loc. cit.*) obtained a digestion product which they showed to be a mixture of free thyroxine with thyroxine still in peptide combination, the latter preponderating. From the mother liquors after the separation of thyroxine, *d*-diiodo tyrosine was subsequently isolated by further action of trypsin and erepsin (Harington and Randall, *Biochem. J.*, 1931, **25**, 1032).

The thyroxine-peptide is much more active physiologically (tested on rats) than laevo-thyroxine when both are given by mouth, but shows the same activity on subcutaneous injection. Iodothyreo-globulin for its iodine content is much more active than racemic thyroxine, given by mouth or subcutaneously, but has the same potency as laevo-thyroxine when both are injected (J. H. Gaddum, *J. Physiol.*, 1930, **68**, 383). The greater activity of thyroxine in peptide combination can be accounted for by the greater solubility

of the peptide and by a difference in the rate of absorption of free and combined thyroxine.

Thyronine (or **Desiodo-thyroxine**), $C_{15}H_{15}O_4N$,

is the amino acid obtained by removal of all the four iodine atoms from thyroxine. Harington found that the best method of doing this was by shaking a 1% solution in *N* potassium hydroxide in an atmosphere of hydrogen in presence of a catalyst of palladium hydroxide and calcium carbonate. It melts (with decomposition) at 252–254°. It forms salts with acids and alkalies, and gives the Millon and ninhydrin reactions. It is entirely devoid of specific thyroid effect.

The hydrochloride crystallises in colourless plates of m.p. 238–240°.

Diiodothyronine, $C_{15}H_{13}O_4NI_2$, has about 1/40 of the activity of thyroxine on tadpoles, and about 1/15 of its effect on the oxygen consumption of rats (Gaddum, *loc. cit.*).

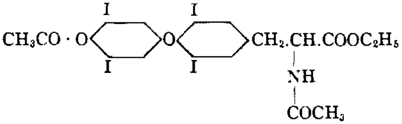
Thyroxine methyl ester hydrochloride, $C_{16}H_{13}O_4NI_4.HCl$, can be obtained from thyroxine by suspending it in methyl alcohol and saturating the suspension with dry hydrochloric acid gas. After cooling, the saturation is repeated, and the solution is concentrated by distillation under diminished pressure. The crystalline precipitate is washed with alcohol and ether, and recrystallised from dilute alcohol containing a little hydrochloric acid. The ester hydrochloride separates in colourless needles of m.p. 221.5° (with decomposition). It is sparingly soluble in water or alcohol, but more so in a mixture of the two.

Thyroxine Methyl Ester is obtained from a solution of the hydrochloride in 50% alcohol by treatment with an equivalent of 2*N* sodium hydroxide solution. A crystalline precipitate forms on addition of water. The ester can be crystallised from dilute alcohol and separates in small prismatic needles of m.p. 156°. It is readily soluble in alcohol, but sparingly soluble in other organic solvents and water.

N-Acetyl Thyroxine, $C_{17}H_{13}O_5NI_4$, is obtained by acetylating the methyl ester, and hydrolysing with 50% alcoholic *N* Sodium hydroxide for 30 mins. at room temperature. It gives an intense nitrous acid reaction. The crystals sinter at 205° and melt (with decom-

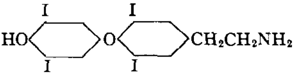
position) at 210–215°. A suspension of the salt was found to be very active physiologically, but when dissolved in sodium hydroxide solution had little effect (Gaddum, *loc. cit.*).

Diacetyl Thyroxine Ethyl Ester,



the "acetyl sulphate" of Kendall and Osterberg (*J. Biol. Chem.*, 1919, **40**, 265), occurs in clusters of colourless needles when precipitated from its solution in a mixture of four parts of alcohol to one part of methyl ethyl ketone by addition of water. It has a melting point of 216–217° after sintering at 213°. The diacetyl derivative gives no nitrous acid reaction and is insoluble in sodium carbonate solution even at the boiling point. This compound is also active physiologically when administered in suspension.

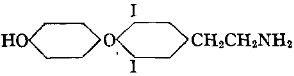
Thyroxamine, $C_{14}H_{11}O_2NI_4$,



is the amine obtained by the removal of the carboxyl groups from thyroxine. It occurs as small colourless needles melting (with decomposition) at 207°.

The sulphate and hydrochloride are very sparingly soluble in water, but dissolve in dilute alcohol.

Diiodothyronamine, $C_{14}H_{13}O_2NI_2$,



forms small colourless rhomboidal crystals melting at 243–245°. It is fairly soluble in alcohol, but insoluble in other common organic solvents and in water.

The hydrochloride forms glistening plates sparingly soluble in cold water, but more readily in dilute alcohol. It melts (with decomposition) at 285–290°.

The sulphate occurs in long fine needles.

It is doubtful whether the amines have a slight physiological effect or none at all.

3:5-Diiodotyrosine is present, together with thyroxine, in the thyroid gland, the total iodine being distributed about evenly between the two compounds. It is found in the acid-soluble fraction of the extract. The differences in solubility, melting point, optical activity and physiological action distinguish it from thyroxine. *d*-3: 5-Diiodotyrosine, the naturally occurring substance, crystallises as straw coloured prismatic needles having a melting point of 194° (with decomposition) and $[\alpha]_{5461} + 3.85°$ in *N*. hydrochloric acid. Like thyroxine, it gives a colour reaction with nitrous acid and ammonia, but it is practically without any physiological effect. Upon evaporation with concentrated hydriodic acid on the water bath the residue gives a strong Millon's reaction.

A large number of other derivatives have been prepared and described, by Kendall and by Harington and their co-workers, but as they have found no commercial application and are not of use in identifying the compound, they have been omitted from this description. Many of them were prepared in the attempt to find a derivative of equal or greater physiological activity but greater solubility, as the uses of thyroxine itself are somewhat restricted by its insoluble nature. The attempt has not yet met with any success. Of synthetic products, glycyl and alanyl thyroxine are most nearly allied to the natural thyreo-globulin, but they are less active and hardly more soluble than thyroxine.

Tests for Thyroxine

The only useful chemical test for thyroxine and the one described in the B.P. 1932 is the nitrous acid colour reaction which is carried out as follows:—A few milligrams are dissolved in 5 c.c. of alcohol, with the addition of 3–4 drops of 50% hydrochloric acid. Five or six drops of 1% sodium nitrite solution are then added, when a yellow colour appears which deepens on boiling. When the solution is cooled and concentrated ammonia added a pink colour is produced. This test is given by a 1 in 40,000 dilution of thyroxine. It is influ-

enced by the presence of impurities, a yellow colour instead of a pink developing with ammonia, or if acetic or sulphuric acid is used instead of hydrochloric, the yellow is not so deep and is turned to orange by addition of ammonia. As the test is given by other *o*-hydroxy diiodo derivatives of benzene, it is not specific for thyroxine, which can only be identified with certainty by physiological test.

The U.S.P. describes the following test:—Ignite a few milligrams with about 0.1 grm. of sodium carbonate, dissolve the residue in 2 c.c. of distilled water, acidify with acetic acid, and add 1 c.c. of chloroform and a few drops of chlorine water. The chloroform acquires a violet colour.

Estimation of Thyroxine

I. CHEMICAL

For many years before the isolation of thyroxine it was known that the efficacy of thyroid preparations depended largely upon the iodine content. Since our present knowledge indicates that thyroxine and diiodotyrosine, with small amounts of inorganic iodides, are the sole iodine-containing compounds present, and that only thyroxine has physiological activity, it is clear that only the estimation of the thyroxine iodine can give any definite idea of the activity of the preparation.

C. R. Harington and S. S. Randall (*Quart. J. Pharm.*, 1929, **2**, 501) recommend the following process for the complete assay of thyroid gland:

1. About 25 tablets (or equivalent quantity if the material is not in tablet form) are ground up, dried, weighed, and suspended in 10 parts of *N* sodium hydroxide solution and boiled under a reflux condenser for 4 hours. Traces of inorganic material are filtered off quantitatively, and the iodine is determined in an aliquot part of the filtrate by Kendall's method (see below).

2. Acid is added to the remainder of the filtrate until a *p*H of 5.0 is reached, and the mixture is set aside for at least 12 hours. The precipitate is filtered off quantitatively, and the iodine in the filtrate is determined.

3. Ten tablets are ground up with 10 c.c. of water and the iodine is estimated in 5 c.c. of the filtrate.

Three different values for the iodine content are thus obtained. The first gives total iodine, the second the acid-soluble fraction (diiodo tyrosine), and the third the inorganic iodine. The thyroxine iodine is found by subtracting the sum of the last two from the first. The weight of pure thyroxine in the original sample can then be calculated.

Kendall's method of iodine estimation (*J. Biol. Chem.*, 1920, **43**, 149) is carried out as follows:—

Not more than 0.5 grm. of dried solid (if the substance to be assayed is in the form of a solution it must first be evaporated to dryness) in a pure nickel crucible (5.9 cm. diameter) is moistened with a few drops of 30% sodium hydroxide solution; 5–10 grm. of stick sodium hydroxide, broken into small pieces, are added, and the excess of water is evaporated on a hot plate, the heating being continued until the contents of the crucible are reduced to a syrup. If there is a tendency to spatter, a small amount of lactose may be added to prevent it. The nickel crucible is then supported inside a larger one of iron (7.8 cm. diameter), the bottom of which is covered with a layer of sand about 0.5 cm. thick. This is held by a cylinder 9.4 cm. in diameter and 30 cm. high, having cross bars to support the iron crucible 7.5 cm. from the top. The burner recommended is a 15.6 cm. No. 3 Meker burner.

The bottom of the iron crucible is heated to redness, great care being taken to avoid excessive heat, which causes volatilisation of some of the iodine. After 5–10 minutes the melt settles to the bottom, but bubbles continue to be given off for five minutes or so. When only a few bubbles are being liberated the small crucible is removed with tongs and partly cooled by moving the contents in the air with a rotatory motion. 5–10 mg. of potassium nitrate are added to oxidise the remaining organic matter. If bubbles appear the addition is repeated again and again until no more are produced. The melt is then poured into the shallow cover of the 5.9 cm. crucible and left to cool. When cool, the lid and crucible are placed in a beaker of 600–800 c.c. capacity, 125–150 c.c. of water are added, and the whole is warmed on the hot plate. When all is dissolved the solution is washed into a 500-c.c. Erlenmeyer flask. The volume should then amount to about 200 c.c. of colourless fluid.

Five c.c. of 20% sodium bisulphite are added and 2 drops of saturated aqueous methyl orange, and the solution is cooled under

the cold tap. Next, 85% phosphoric acid is added slowly, the flask being vigorously shaken to expel carbon dioxide. The addition of the acid is stopped when the indicator changes to a pink colour, and 5 c.c. of 20% phosphoric acid and a small piece of hard coal (about 0.5 cm. diameter) are added. The volume is adjusted to about 250 c.c., and the solution is boiled until it is reduced to about 200 c.c. The flask is then cooled and bromine is added, with continual shaking, until the solution is definitely yellow in colour. It is then boiled until the colour has disappeared, when the boiling is continued for another 5 minutes. A few crystals of salicylic acid are added, and the flask is cooled in cold water; the volume should not be less than 175 c.c. Five c.c. of reduced 20% phosphoric acid and about 1 grm. of pure potassium iodide crystals are added, and the liberated iodine is titrated with 0.005 *N* sodium thiosulphate solution. A 0.5% solution of soluble starch is recommended for use as an indicator at the end of the titration.

Recently a method for thyroxine estimation which offers many advantages was presented to the Society of Public Analysts by G. Middleton (*Analyst*, 1932, **57**, 603). This involves an improvement in the method of determination of iodine, which is assayed directly in the thyroxine fraction. The result therefore depends upon one analysis of a high degree of accuracy, instead of upon a difference between two figures each of which is subject to a larger error.

The method is as follows:—Hydrolysis and precipitation of the acid-insoluble (thyroxine-containing) fraction are carried out as in the method of Harington and Randall. The precipitate is then collected on a 45 mm. filter paper in a specially constructed apparatus, whereby the tedious nature of a long filtration is avoided. The filter paper and precipitate are dried at 100° in the small crucible in which they are to be ignited. Ignition is carried out as follows:—The crucible is filled with dry powdered sodium carbonate and inverted on to a layer of it at the bottom of a large crucible of 36 mm. diameter. More sodium carbonate is added to fill the space between the two crucibles, which are then heated for 20 minutes so that the outside of the large one assumes a dull red heat. After cooling, the contents are tipped into a beaker containing about 100 c.c. of water and broken up immediately, and the larger crucible is rinsed out. After being boiled for ten minutes the solution is filtered into a 600-c.c. conical flask through a filter paper which has

been hardened by momentary immersion in strong nitric acid and washing. The residue on the filter paper is washed back into the beaker by means of a jet of water, and the extraction completed by boiling with more water for 30 minutes. The filtrate and washings are made up to about 350 c.c., and methyl orange is added followed by 50% sulphuric acid until the mixture is just acid. Ten c.c. of 10% sulphuric acid are added in excess, and a few drops of iodine-free bromine. The solution is heated, and when it commences to boil, a current of carbon dioxide is passed in to prevent bumping. When the solution has only a slight yellow colour (after about 3 minutes' boiling) it is cooled, and about 0.2 c.c. of a 25% solution of crystalline phenol in glacial acetic acid is added. After standing for at least two minutes a few crystals of potassium iodide are added, and the mixture is titrated with $N/100$ sodium thiosulphate solution, with starch as indicator.

Thyroxin-sodium is assayed in the British Pharmacopoeia 1932 by dissolving in sodium hydroxide solution and evaporating and fusing in a nickel crucible after the addition of a small amount of albumen. To complete the oxidation of organic matter occasional additions of potassium nitrate are made. Finally, the whole is dissolved in water, a few drops of methyl orange solution are added, followed by a definite amount of sodium metabisulphite solution, and phosphoric acid until the solution is faintly pink. The iodide in solution is then oxidised to iodate by boiling with an excess of bromine, the excess of bromine being removed by the addition of a little sodium salicylate. After cooling, potassium iodide solution and phosphoric-acid are added and the liberated iodine is titrated with thiosulphate solution.

For the assay of thyroid, a weighed amount is boiled under a reflux condenser with sodium hydroxide solution, the solution is filtered and, after dilution to known volume, an aliquot portion of the total filtrate is evaporated to small volume on the water bath and the assay is carried out in exactly the same way as for thyroxin-sodium, but without the addition of albumen. This gives the total iodine. Another portion of the filtrate is acidified with sulphuric acid, and the iodine is estimated in the solution after filtration. The difference between the results of these two estimations gives the thyroxine iodine.

The inorganic iodine is obtained by extracting thyroid with cold water and estimating the iodine in the filtrate.

The methods of the German (D.A.B.VI) and United States (Xth Decennial Revision) Pharmacopoeias involve fusion with sodium and potassium carbonates and potassium nitrate, and subsequent oxidation with potassium permanganate, or chlorinated soda.

Many commercial preparations of thyroid gland are stated to be equivalent to so many grains of fresh thyroid, but it has been shown by Kendall and by Harington and Randall that this may be very misleading, since even if the content of thyroxine were proportional to the iodine in the gland (and it has been shown that this is not so), there is still a marked variation in the iodine content of the gland. Thus, in a recent series of estimations it was found that the total iodine varied from 0.21 to 0.78%, and the thyroxine iodine from 0.027 to 0.19% of the dried gland. The thyroxine iodine was 12 to 37% of the total. In Table I are given the variations found by Harington and Randall (*loc. cit.*) in the organic, thyroxine, and inorganic iodine contents of a number of commercial samples.

TABLE I

Sample	Organic iodine in dried gland	Thyroxine iodine in dried gland	Total organic iodine as thyroxine	Inorganic iodine in dried gland	Total iodine as inorganic iodine
	%	%	%	%	%
A	0.106	0.03	28.0	0.006	5.2
B	0.20	0.12	60.5	0.024	10.7
C	0.16	0.08	51.5	0.008	4.9
D	0.36	0.15	43.2	0.016	4.2
E	0.23	0.11	48.7	0.034	13.0
F	0.17	0.09	52.3	0.008	4.3
G	0.26	0.10	37.7	0.001	4.5
H	0.32	0.19	59.5	0.025	7.2

These figures are calculated on the assumption that each tablet contains 65 mg. (1 grain) of dried gland.

Thyroid gland loses about 75% of its weight on drying. The weight of a fresh sheep's gland (two lobes) is about 4 grm.

As a result of this work Harington and Randall suggest that thyroid gland should be standardised to contain 0.09 ± 0.01% thyroxine iodine, and that the inorganic iodine present should not exceed 10% of the total. Lactose may be used to dilute powders of greater thyroxine content.

2. PHYSIOLOGICAL

Although thyroxine is a pure chemical compound, it is frequently used therapeutically in the form of dried thyroid gland, which, in addition to thyroxine in peptide combination with protein, contains diiodotyrosine. The combined thyroxine, being more soluble than the pure laevo compound, has a greater physiological activity when given by mouth. Estimations of thyroxine iodine are then insufficient if part of the thyroxine is not combined in peptide linkage. It is therefore advantageous to carry out a physiological test of the potency of dried thyroid gland in addition to the usual chemical estimation.

Available methods depend on the ability of thyroxine to increase the resistance of mice to acetonitrile, and on its stimulant effect on the metamorphosis of amphibian larvae, or on the metabolism of mammals. None of these tests shows the same degree of quantitative accuracy that can be attained in tests on adrenaline or insulin.

The Health Organisation of the League of Nations recommend the acetonitrile test and the use of a preparation of dried thyroid gland containing 0.2% iodine in specific combination as standard (E. Knaffl-Lenz. *Publications of the League of Nations, III. Health,* 1928, III. 10. C.H. 734, p. 35).

1. **Acetonitrile Method.**—Male mice from a uniform stock, of 15 to 20 grm. in weight, kept at a temperature of 18–20° and on a constant diet, are divided into two groups. One is deprived of food for 4 hours; half of this group are then given by stomach tube 5 mg. and the other half 10 mg. of dried and powdered thyroid in watery suspension. 0.2 c.c. per 20 grm. body weight of a 2.5% or 5% suspension is conveniently administered. The animals are fed the same afternoon. Twenty-four hours later all the mice are deprived of food for four hours, and are then injected intravenously or subcutaneously with a freshly prepared 9% solution of acetonitrile in 0.9% sodium chloride solution. The animals which have not received thyroid should be divided into three groups and given, respectively, doses which are 75%, 100% and 125% of the estimated lethal dose of acetonitrile, which is about 0.9 mg. per grm. of mouse. For the animals which have received thyroid the doses of acetonitrile should be from 1.5–2.5 times the estimated lethal dose. After the

administration of 10 mg. of the standard dried thyroid the resistance to acetonitrile should be increased 100%. Reid Hunt (*U. S. Public Health Reports*, 1925, **40**, 1461) recommends that dosage of an unknown preparation should be based on its iodine content. It is then easy to observe whether the sample has been adulterated by adding inorganic iodine, since the protection against acetonitrile is much less than that expected. The error of the test is about 30%. Greater accuracy would probably be attained by constructing a dose-mortality curve for acetonitrile for the stock of mice available, and another for the same stock after administration of a dose of thyroxine or dried thyroid gland, which increases the resistance of the animals by 100%. These curves can then be used in the assay of other samples. The standard should be given to a group of mice at the same time as the sample under test, to control variations in the sensitivity of the stock of animals.

2. **Tadpole Method.** (J. H. Gaddum, *J. Physiol.*, 1927, **64**, 246).—Batches of 12 tadpoles are kept in 500 c.c. tap water which is changed daily, and fed on hard-boiled white of egg. Each batch is immersed for 24 hours in 500 c.c. of a dilute solution in tap water of the material under test. They are then transferred again to fresh water. The decrease in length of the tadpoles is roughly proportional to the amount of thyroxine in the solution. For dried thyroid the animals can be fed with this material, but the test is less satisfactory. The effect can be compared with that produced by the standard on batches of tadpoles kept under similar conditions.

3. **Mouse Metabolism Method.** (J. R. Mørch, *J. Physiol.*, 1929, **67**, 221).—Male mice weighing 16–22 grm. are fed on a constant diet. A suitable mixture is crushed corn 200 grm., crushed barley 200 grm., dried milk 80 grm., of which 4 grm. together with 3 c.c. of fresh milk are given daily. After 8 days the mice are placed each in a jar immersed in a water-bath maintained at a temperature of 23°. Air freed from carbon dioxide is drawn through the jar, and the carbon dioxide given off by the animal is collected in potassium hydroxide solution. The output for 24 hours is determined for four or five consecutive days, and the mean forms the normal value for carbon dioxide production. The thyroid preparation is given mixed with the food, in doses of 0.1–1 mg. per 20 gram mouse in the case of dried thyroid. After three weeks the carbon dioxide output is again determined on five consecutive days. Allowance

must be made for loss or gain in weight of the animals. If they have gained in weight, the carbon dioxide output is expressed in sq.cm. of surface (surface = $11.36 \times (\text{weight})^{2/3}$), if they have lost weight, per gram of body weight. The increase in metabolism with varying doses bears a roughly linear relationship to the logarithm of the dose given. The most accurate results are obtained when the increase in metabolism is not greater than 30%. Doses of thyroid differing from each other by 30% can be distinguished.

A similar method was applied to rats by J. H. Gaddum (*J. Physiol.*, 1930, **68**, 383) in which the oxygen consumption was estimated instead of the carbon dioxide output.

Uses and Toxicology

Thyroid gland or thyroxine is employed in all conditions in which there is a deficiency of the natural secretion of the gland, *e. g.* simple goitre, myxoedema, cretinism. Its action as a stimulant of metabolism has led to its use in obesity, in certain skin diseases, especially psoriasis, in chronic rheumatic infections, and disorders of menstruation in women. It has also been used to reduce oedema and as a general tonic.

Signs of overdosage appear only after several days, and include quickening of the pulse, feverishness, flushing of the skin and sweating, headache, nausea, vomiting, diarrhoea, sleeplessness, tremors, pruritus, and sometimes delirium. Signs of chronic overdosage may appear in the course of prolonged treatment, and include emaciation, muscular weakness, loss of hair, dilatation of the pupils, and general debility. In fatal cases, uncontrollable vomiting precedes collapse and coma. Its use is contra-indicated in exophthalmic goitre.

PHARMACOPOEIAL PREPARATIONS

Thyroid is official in the British Pharmacopoeia, 1932. Synonymous terms are thyroideum siccum, dried thyroid, thyroid extract, thyroid gland.

It should contain 0.1% of iodine (0.09–0.11%) in combination as thyroxine, and not more than 10% of the total iodine should be present as inorganic iodine.

It is prepared from thyroid gland by drying the tissue and extracting the fat. It is a cream-coloured amorphous powder with a faint meat-like odour and taste. If necessary, it can be mixed with

lactose to reduce the thyroxine iodine content to 0.1%. A test for identity is described.

According to the German Pharmacopoeia (D. A. B. VI) it is possible to recognise dried and powdered thyroid gland by microscopic examination. Under the low power yellowish gray or reddish yellow masses are observed, composed chiefly of gland vesicles; isolated portions of colloid, however, are structureless and translucent, and show a conchoidal surface of fracture. The vesicles are seen more clearly under the high power after treatment with dilute caustic potash: they are lined with flat or cubical cells and contain colloid, and are surrounded with blood vessels; elastic fibres from the capsule of the gland may also be observed among them.

There should be present 0.18% of organic iodine, and yeast cells, lactose, starch, salts, etc., should be absent. Treatment with iodobenzene, followed by washing with petroleum benzene should stain the powder uniformly brown, and no colourless particles should be observed under the high power of the microscope (×300). One grm. of dried gland should not yield to aqueous alcoholic and ethereal extractions, carried out successively, more iodine than can be titrated with 0.1 c.c. of 0.1 *N* sodium thiosulphate solution.

The U. S. Pharmacopoeia (Xth Revision) specifies between 0.17 and 0.23% of iodine in thyroid combination, together with freedom from inorganic iodine or any type of iodine other than that peculiar to the thyroid.

The average dose is 0.06 grm. (U. S. P. X), the greatest single dose 0.5 grm. (D. A. B. VI). In the B.P. 1932 the dosage given is 0.03 to 0.3 grm.

Thyroxine is official in U. S. P. X. and as thyroxinsodium in the B.P. 1932. The average dose is 0.5 mg.

Both thyroid gland and thyroxine can be given by mouth, as no destruction takes place in the intestinal tract. Thyroxine can also be given by injection.

THE HORMONE OF THE PARATHYROID GLANDS

The parathyroid glands are small bodies found close to, or embedded in, the lateral lobes of the thyroid. Their position and number vary considerably in different animals; usually there are two on each side. They have not been found below the amphibia. They are developed from the epithelium of the third and fourth

branchial clefts on each side. They consist of small epithelium-like cells arranged in a compact or lobular manner. The cells are either clear or granular in appearance from the presence of oxyphil granules. The parathyroid glands secrete a hormone which is essential for the normal metabolism of calcium in the body. It is generally referred to as the parathyroid hormone.

It has not yet been isolated but is administered in the form of an extract obtained from ox parathyroid glands, the potency of which is determined by biological tests. In the absence of a standard, units have been defined in terms of an animal reaction. Collip's unit is 1/100 of the amount of the extract under test required to produce a rise of 5 mg. in the serum calcium of a 20 kg. dog in 15 hours (J. B. Collip and E. P. Clark, *J. Biol. Chem.*, 1925, **64**, 485). Hanson's unit is 1/100 of the amount required to produce each mg. rise in serum calcium in a 15 kg. dog 24 hours after the operation of parathyroidectomy, when given in sufficient amount to produce a rise of 3 mg. in 6 hours (A. M. Hanson, *J. Amer. Med. Assoc.*, 1928, **90**, 747).

Preparation

The active principle of the parathyroid glands appears to bear a definite resemblance to insulin, although it is somewhat more stable. The method of extraction is similar, but much less work has been carried out on its purification, and without a doubt the active extract employed in therapeutics, like insulin in the early days of its manufacture, contains a large proportion of inert protein.

The following is the method of extraction described by Collip and Clark (*J. Biol. Chem.*, 1925, **66**, 133). Fresh parathyroid glands are ground in a meat chopper and extracted with an equal volume of 5% hydrochloric acid, or, if the glands have been preserved in dry acetone, 3% acid is used. The mixture is heated on the boiling water bath for ½–1 hour, diluted with 4 volumes of hot water and allowed to stand. The fat which separates on cooling is removed. Practically all the supernatant material is then dissolved by addition of sodium hydroxide solution to *p*H 8–9. Hydrochloric acid is then added, with stirring, until maximum precipitation occurs (*p*H 5.5–6.5). The precipitate is filtered off and dissolved on the filter with weak sodium hydroxide solution. It is again precipitated by addition of hydrochloric acid, and this process is repeated so long as

any precipitate can be salted out of the filtrate when acidified to congo red. Finally the active fraction is salted out of the filtrate by saturation with sodium chloride after acidifying to congo red, the first filtrate being treated separately. The precipitate, after filtration, is dissolved in weak sodium hydroxide, and the solution is centrifuged and adjusted to *p*H 4.8. The precipitate is separated by centrifuging, dissolved in hydrochloric acid, and the precipitation at *p*H 4.8 is repeated until the mother liquors are clear. After filtration of the acid solution through a Berkefeld filter and adjustment of the *p*H to about 3.6, it is ready for use.

The solid can be obtained from the extract by suspending the precipitate produced at *p*H 4.8 in absolute alcohol, and adding an equal volume of dry ether. The suspension is centrifuged, washed with dry ether, and dried in air.

More recently Tweedy has published a modification of this method (W. R. Tweedy, *J. Biol. Chem.*, 1930, **88**, 649) which involves a somewhat different means of purifying the acid extract.

1. As much fat as possible is removed from fresh frozen glands, which are ground in a meat chopper, transferred to a flask, and treated with 200 c.c. of 3% hydrochloric acid per 100 grams on the water-bath at 70° for 20 minutes.

2. The extract is cooled in a stream of cold water and poured through several folds of cheese cloth to remove fat and undissolved material.

3. Weak sodium hydroxide solution is added until the solution is slightly alkaline to congo red.

4. An equal volume of redistilled acetone is added, and the mixture is allowed to stand.

5. It is filtered through a coarse filter paper, and the filtrate is evaporated under reduced pressure at 50° to one-third of its volume. Two volumes of water are then added, and a freshly prepared solution of trichloroacetic acid to give a total concentration of 2½%. The mixture is cooled on ice, and the precipitate is separated by decantation and centrifuging.

6. The precipitate is stirred up with a small amount of alcohol, transferred to a desiccator, and dried *in vacuo* at room temperature, and afterwards over calcium chloride.

7. The dry residue is ground to a powder and extracted with redistilled chloroform in a Soxhlet apparatus.

8. The powder is freed from chloroform and dried.

Properties

As extracted by Tweedy, the hormone occurs as a gray amorphous powder, partly or entirely soluble in 0.9% sodium chloride solution. Collip describes the following properties, but, as the extracted material is obviously an impure mixture, no importance should be assigned to the protein colour reactions obtained. It gives the xanthoproteic, Millon, biuret, ninhydrin, Hopkins-Cole, and Pauly reactions. Sulphur and iron are present, phosphorus absent. It is soluble in absolute alcohol to the extent of 0.1%, insoluble in acetone, ether, benzene, ethyl formate, ethyl butyrate, chloroform, methyl salicylate. It is insoluble in 100% pyridine, but after solution in 10% acetic acid it is not precipitated by addition of pyridine. It is insoluble in anhydrous acetic acid, but soluble in 98%, and freely soluble in 94%, from which solution it is almost completely removed by addition of 2–3 volumes of acetone.

Solutions are comparatively stable, but the physiological activity is destroyed by boiling for one hour in 10% hydrochloric acid or 5% sodium hydroxide solution. It is stable in phenol up to 120°. Tweedy found that a solution in phenol, when kept at 100° for 10 days, did not lose more than about half its activity. It is destroyed by pepsin and trypsin, and completely inactivated by ultra-violet irradiation. A suspension in absolute alcohol is inactivated by hydrochloric acid gas. The active principle does not dialyse through collodion to any appreciable extent. It is adsorbed by norite or the Folin-Wu tungstic acid precipitate.

Hydrochloride.—The hydrochloride may be obtained by suspending the hormone in acid alcohol (15 c.c. of 10% hydrochloric acid to 85 c.c. of 95% alcohol) and precipitating it with ether; the precipitate is washed with alcohol and ether on the centrifuge. The hydrochloride is a white powder easily soluble in 0.9% sodium chloride.

Picrate.—The picrate is soluble in 70% acetone, and can be converted to hydrochloride by the same process that was used by Dudley for insulin (see p. 387). The picrate is insoluble in water.

Physiological Assay

No standard is yet available and the tests are only roughly quantitative. Normal or parathyroidectomised dogs are used.

1. Normal dogs. (J. B. Collip, *Medicine*, 1926, **5**, 1; W. R. Tweedy, *J. Biol. Chem.*, 1930, **88**, 649).—Food is withdrawn from the animals after a meal in the morning, and a sample of blood taken in the afternoon, and the calcium content of the serum estimated by the method of Kramer and Tisdall (*J. Biol. Chem.*, 1921, **47**, 475) as modified by Collip and Clark (*J. Biol. Chem.*, 1926, **63**, 461). A subcutaneous injection of the extract under test is then given and a second sample of blood withdrawn the next day, 15 or 16 hours later. The increase in serum calcium is almost directly proportional to the dose given. A number of different dogs are used on a series of doses. Animals show fairly constant responses to the same dose; *they should be given several days rest between tests. The amount* of extract given should be such as to raise the serum calcium to about 14–16 mg. per 100 c.c., that is, from 50–200 units.

2. Parathyroidectomised dogs. (A. M. Hanson, *J. Amer. Med. Assoc.*, 1928, **90**, 747). Twenty-four hours after the operation of parathyroidectomy the serum calcium has fallen 30%. Following the injection of an active extract the serum calcium rises to or above normal, and for a series of about six dogs the average rise is roughly proportional to the dose given.

Uses and Toxicology

Parathyroid extract, on subcutaneous injection, produces a rise in the calcium of the blood. It is therefore used in any condition in which this is reduced. It is a specific in tetany, especially that due to removal of parathyroid tissue in the operation of thyroidectomy. It has also been used to stimulate the excretion from the bones, and thus from the body, of lead and radium in lead or radium poisoning. It has been employed in haemorrhage, since calcium plays an important part in blood clotting, as a diuretic, and in certain nervous disorders, such as epilepsy.

Overdosage produces an excessive rise in the calcium of the blood, which leads to vomiting and haemorrhage in the intestinal tract. There may be headache, nausea, and rapid heart beat. The onset of vomiting and diarrohea when parathyroid extract is being given is a warning that treatment should be stopped. The extract is not effective when given by mouth, and dried parathyroid gland has no definite value in therapeutics.

Parathyroid extracts for subcutaneous, intramuscular, or intravenous injection, are described in "*New and Non Official Remedies*" (Amer. Med. Assoc., 1931, Chicago, p. 304).

ADRENALINE

Synonyms: Epinephrine. Suprarenine. Adrenine.

Adrenaline is the internal secretion of the medullary portion of the suprarenal (or adrenal) glands. These glands are situated in the neighbourhood of the kidneys. In mammals they are paired capsules consisting of an outer cortex and a central medulla, and lie in close relationship with the upper pole of each kidney. In birds, reptiles and amphibians, the two portions of the gland are interlocked with the renal tissue, whilst in elasmobranchs the two parts remain separate, the cortex being represented by the inter-renal body and the medulla by paired bodies lying close to the ganglia of the sympathetic chain.

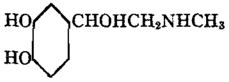
The development and functions of the two parts of the gland are entirely different. The cortex is developed from the mesoderm cells of the genital ridge. It is composed of polygonal epithelium cells containing lipoid granules and a certain amount of pigment. It secretes a hormone which is essential to life, and in the absence of which a condition known as Addison's disease (in man) develops.

The medulla is formed from the same primitive nervous tissue that gives rise to the nerve cells of the sympathetic ganglia. It consists of irregular anastomosing columns of cells with blood sinuses between them. The cells contain granules which are stained brown with chromic acid and its salts; they secrete adrenaline.

Adrenaline is obtained from the glands of sheep or other animals or is prepared by synthesis. It is present in the venom of the toad (*Bufo agua*) and has been detected physiologically in blood.

Constitution

Adrenaline, $C_9H_{13}O_3N$, is dihydroxyphenylmethylaminoethanol.



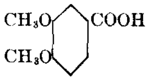
Its constitution is shown by the following reactions and proved beyond all doubt by the method of its synthesis.

1. Fusion with potassium hydroxide gives rise to catechol and protocatechuic acid.

2. Heated with acids or sodium hydroxide, methylamine is liberated.

3. The action of phenylisothiocyanate shows the presence of a secondary amino group.

4. Methylation with methyl sulphate followed by oxidation with potassium permanganate gives veratric acid



and trimethylamine. The methyl imide group—$NH.CH_3$ is likely to be in the side chain because it is readily liberated.

5. Benzoylation shows the presence of three OH groups, one of which must be in the side chain.

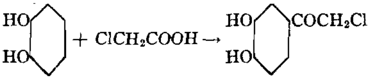
6. Adrenaline is optically active and must therefore contain an asymmetric carbon atom.

7. Tribenzene sulphonyl adrenaline when oxidised to the corresponding keto compound loses its optical activity; the OH group of the side chain must therefore be attached to the asymmetric carbon atom.

Preparation

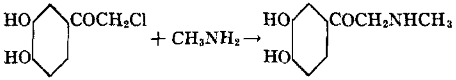
1. SYNTHESIS

1. Catechol condensed with monochloracetic acid in presence of phosphorus oxychloride gives chloracetocatechol:



Ten parts of pyrocatechol are mixed with 8 parts of monochloracetic acid and the mixture is carefully heated. When the evolution of hydrochloric acid commences, the heating is discontinued, since the reaction will proceed to completion. The chloracetocatechol may be recrystallised from water after being decolourised with charcoal. It separates as colourless needles of m.p. 173°.

2. Adrenalone is formed from chloracetocatechol by treatment with methylamine:



The chloracetocatechol is finely ground and suspended in one part of alcohol, and one part of aqueous 60% methylamine is added. The solution is maintained at a moderate temperature and methylamino-acetocatechol separates. After standing for one hour the crystalline precipitate is filtered off, washed with cold alcohol, and purified by dissolving in dilute hydrochloric acid and adding a small amount of ammonia. This causes precipitation of a small quantity of an amorphous impurity before the base itself is precipitated. Racemic methylamino-acetocatechol is precipitated in the form of clear yellow crystals of m.p. 230° (with decomposition) after darkening at 200°. The hydrochloride separates from alcohol in colourless leaflets which decompose at 240°.

3. Racemic adrenaline is obtained from methylamino-acetocatechol (or adrenalone) by reduction. One part of adrenalone is dissolved in 30 parts of water containing the calculated quantity (half mol.) of sulphuric acid. The solution is heated on the water bath, and one part of a 1% solution of mercuric sulphate together with one part of powdered aluminium is added. After 3–4 hours' heating, during which time it must be stirred, the base separates, but is kept in solution by successive small additions of sulphuric acid. Excess of acid and aluminium are precipitated by neutralising with baryta and are filtered off. The solution is evaporated to dryness *in vacuo* when adrenaline sulphate remains as an amorphous mass which may be purified by solution in water and precipitation with ammonia. Adrenaline is precipitated in crystalline form and is filtered off.

4. Resolution is carried out by means of fractional crystallisation of the bitartrate from methyl alcohol.

50 grm. of *dl*-adrenaline are moistened with absolute methyl or ethyl alcohol, and dissolved in an alcoholic solution of 43 grm. of *d*-tartaric acid. The mixture is taken to dryness *in vacuo*, heating to 35–40°. The dry tartrate is ground up with a small amount of methyl alcohol when the *d*-adrenaline *d*-tartrate dissolves, the *l*-salt being less soluble. The residue which consists mainly of *l*-adrenaline *d*-tartrate is purified by recrystallisation from methyl

alcohol until it has a melting point of 149°. The *d*-adrenaline can be recovered from the mother liquors after concentration by precipitation with ammonia, racemised by treatment with mineral acid and separation again effected. Thus it is possible ultimately to obtain the whole as *l*-adrenaline. The two enantiomorphs can also be separated by means of the mould-fungus *Penicillium glaucum*.

2. EXTRACTION

The fresh glands are minced and repeatedly extracted with boiling acidified water with the addition of a little zinc dust. The combined extracts are filtered and evaporated *in vacuo* at 50° to a syrup. The concentrate is mixed with several volumes of ethyl or methyl alcohol and treated with lead acetate solution until no further precipitation occurs. The filtrate is freed from lead by means of hydrogen sulphide and evaporated in a current of carbon dioxide. When it has been reduced to a syrup, a layer of paraffin is added and excess of concentrated ammonia. Sulphites may with advantage be kept present during all operations to prevent oxidation. Inorganic impurities may be removed from the crude base by grinding with a strong solution of oxalic acid in 90% alcohol, which dissolves the adrenaline. The solution is filtered and the adrenaline is re-precipitated with strong ammonia.

The above method was originated by Aldrich. Other methods differ in the way of separation of proteins from the acid extract.

In Takamine's method the finely minced glands are extracted for about five hours with acidified water at 50–58°, the mixture being stirred and water constantly added to replace losses by evaporation. To coagulate the proteins the temperature is raised to 90–95°, keeping a layer of fat on the surface. The liquid is then pressed out and the extraction repeated. The combined extracts are concentrated and 2–3 volumes of alcohol added to precipitate inert substances. The alcohol is removed *in vacuo* and ammonia is added. After standing for several hours the precipitate is filtered off, washed, and dried.

In Abel's method the minced glands are mixed with half their weight of alcohol containing 3–4% trichloroacetic acid. The solution is filtered after standing, and the filtrate is evaporated and treated with ammonia. The crystalline precipitate is purified by

dissolving it in dilute oxalic acid solution, precipitating with a mixture of alcohol and ether, and re-dissolving in water containing trichloroacetic acid. 800 c.c. of absolute alcohol together with 150 c.c. of ether are added to each 50 c.c. of acid extract: the solution is filtered and the adrenaline is precipitated with ammonia.

In Weidlein's method, extraction from the gland of the whale is carried out as follows:—to one kg. of minced glands 550 c.c. of absolute alcohol, 50 c.c. of chloroform, and 25 c.c. of acetic acid are gradually added. The extraction is continued for about 12 hours when the proteins are coagulated by warming and filtered off. About 600 c.c. of filtrate are obtained. This is evaporated *in vacuo* to about one-tenth of its volume, ammonia is added, and the precipitate is filtered off and dried with alcohol and ether. Weidlein reports a yield of 0.15% of adrenaline.

Other variations of these methods include extraction with oxalic or acetic acids and precipitation with methylamine. In all, the essentials are to exclude air as far as possible and to avoid prolonged heating with mineral acids, which causes racemisation. As the *d*-base has only 1/15 of the activity of the natural *l*-adrenaline, this is most undesirable.

Purification is best effected as follows:—Five grm. of nearly pure adrenaline are dissolved in 25 c.c. of 85% alcohol containing 3.5 grm. of pure oxalic acid. About 15 c.c. of dry ether are added until the solution is slightly turbid, then enough ammonia in ether and alcohol mixture to precipitate the inorganic impurities. These are filtered off and the base precipitated by aqueous ammonia. The precipitate is washed thoroughly and dissolved in very dilute sulphuric acid, two volumes of 96% alcohol and ether being added. After standing for half an hour the solution is filtered and the adrenaline again precipitated from the filtrate. It is washed thoroughly, dissolved in dilute hydrochloric acid and an equal volume of alcohol added, excess of acid being removed by addition of ammonium acetate. A few drops of ammonium oxalate and dilute ammonia are added until the solution is weakly alkaline. If any turbidity is seen, the adrenaline is again precipitated, dissolved in hydrochloric acid, and treated with very dilute ammonia, the precipitation being repeated three times. This process yields an ash-free material (3.88 grm. from 5 grm. of almost pure adrenaline).

Properties

dl-Adrenaline, m. p. 208°.

l-Adrenaline, m. p. 211° $[a]_D$ −53° in dilute hydrochloric acid.

According to Takamine, adrenaline crystallises in five different forms, but usually separates as tufts of colourless needles. Adrenaline is the most powerful of the naturally occurring substances which have the property of raising the blood pressure upon injection. It has a slightly bitter taste and produces numbness of the tongue. It is a weak base and is alkaline to litmus and phenolphthalein. Natural adrenaline is laevo-rotatory and far more powerful and more toxic than the dextro-form.

The solubility in cold water is 0.0268% at 20°; it is slightly greater in hot water. Adrenaline is soluble in warm ethyl oxalate, benzaldehyde, and glacial acetic acid, but not in ordinary organic solvents. It is readily soluble in aqueous solutions of mineral acids and sodium or potassium hydroxides, but not in aqueous solutions of ammonia or alkali carbonates. It is precipitated from solution in acids by ammonia; it is not precipitated by the usual alkaloidal precipitants, but by ammoniacal solutions of lead or zinc salts. Adrenaline is unstable in neutral and alkaline solutions but is stable when dry. It is easily oxidised especially in alkaline solution or in presence of ferrous salts with production of characteristic colour changes. Silver is deposited from ammoniacal silver nitrate in the cold, and gold from gold chloride solutions. Upon oxidation the absorption spectrum in the ultra-violet is displaced in the direction of the visible spectrum. The following values have been given for the specific rotation (*The Chemical and Physiological Properties of the Internal Secretions:* E. C. Dodds and F. Dickens, p. 165):—

	Rotation	Temperature
Bertrand (1904) Natural adrenaline	−53.5°	....
Abderhalden and Guggenheim (1908) Natural adrenaline	−50.72°	20°
Taveau, quoted by Schultz (1909) Natural adrenaline	−51.4°	26.4°
Flächer (1909) *d*-Adrenaline	+51.88°	19.6°
l-Adrenaline	−51.40°	19.8°
Weidlein (1912) Whale adrenaline	−52.0°	25°
Abel and Macht (1912) *Bufo agua* adrenaline	−51.3°	20°

Most salts are amorphous and deliquescent, but the racemic hydrochloride and oxalate are crystalline whilst the corresponding dextro salts are amorphous. Adrenaline gives a stable urate, oxalate, hydrochloride, and bitartrate.

SALTS OF ADRENALINE AND RELATED SUBSTANCES

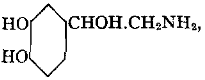
Adrenaline hydrochloride.—The racemic salt is prepared by moistening the base with absolute alcohol and dissolving it in absolute alcohol containing the calculated quantity of hydrochloric acid. On standing, the hydrochloride crystallises. It may be recrystallised from absolute alcohol m. p. 157°. It is readily soluble in water.

Adrenaline acid tartrate, m. p. 149°, is a white powder, soluble in water.

Adrenaline borate is said to be more stable than other salts of adrenaline.

A number of substitutes has been prepared containing the same type of chemical configuration. Of these the most important are the following:—

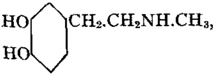
Arterenol,



has m. p. 191°. The racemic form is more active than racemic adrenaline. It can be obtained by reduction of aminoaceto-catechol, or of the cyanhydrin of protocatechuic aldehyde with sodium amalgam.

The hydrochloride melts at 141°.

Epinine,



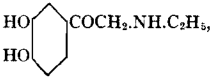
m. p. 188–189°, is obtained in crystalline form on addition of ammonia to an aqueous solution of the hydrochloride. It crystallises from absolute alcohol in colourless clusters of transparent spikes. It is sparingly soluble in cold water but more readily in hot, and only slightly soluble in boiling alcohol. It has a very slight solubility in cold alcohol and other organic solvents. It has about one-tenth the activity of racemic adrenaline.

The ethyl and propylamines have some pressor effect but are less active than the methylamine.

Epinine hydrochloride occurs as long colourless prisms of m. p. 179–180°.

Epinine sulphate occurs as colourless transparent prisms of m. p. 289–290°.

Hormorenol,



is less active than epinine.

Adrenalone, the ketone corresponding to adrenaline formed by its oxidation, is a crystalline substance, melting at 230° (with decomposition). It has a slight physiological activity. The hydrochloride crystallises from alcohol in colourless leaflets of m. p. 240°.

Tests for Adrenaline

Oxidising agents give a pink or rose red coloration. A large number of colour tests has been described, but none is absolutely conclusive, because the reactions on which they depend are given by other related substances. It is therefore necessary always to confirm the test physiologically and by determination of the rotation.

1. *Iodine or iodic acid.* After addition of the reagent, excess should be removed by shaking with ether, when it is possible to detect adrenaline in a dilution of 1 in 1½ million. Fränckel and Allers recommend the addition of an equal volume of $N/1,000$ potassium bi-iodate and a few drops of dilute phosphoric acid (sensitive to a dilution of 1 in 300,000). Bayer has modified the test by adding sulphanilic acid or α-naphthylamine. The sulphanilic acid increases the sensitivity, intensity and duration of the coloration, but it should not be added in the case of the simple iodine test, since it then gives a similar colour to that given by other aromatic substances. The limiting dilution is 1 in 5 million.

2. *Sodium or potassium persulphate.* A solution of a persulphate is added until the concentration is about 1 in 1,000. The mixture is then warmed on the water bath for a short time, when a red colour develops. The sensitiveness of this test is about 1 in 5 million, and it is claimed that the colour is not interfered with by the colour of extracts to the same extent as the Bayer-Fränckel-Allers test described above. It is also said that it is not interfered with by the presence of proteins. The test has been modified by Moreschi as

follows:—A minute amount of tincture of iodine is carefully floated on to the surface of the liquid to be tested. A pink colour develops at the zone of contact if adrenaline is present. This spreads and disappears, but on adding a few drops of 1 in 1,000 aqueous sodium persulphate, a reddish or violet colour reappears. It is claimed that this test is sensitive in a dilution of 1 in 2 million, and has the advantage that the colour persists for several days.

3. *Mercuric chloride.* Comessatti describes the test with mercuric chloride solution by which adrenaline may be distinguished from catechol. To 1 c.c of adrenaline solution (1 in 100,000) add 1 c.c. of sodium acetate solution and 4–5 drops of 1 in 1,000 aqueous mercuric chloride. (The reaction is catalysed by metallic salts of weak acids.) A pale rose colour appears in about 5 minutes at the ordinary temperature in dilutions up to 1 in 400,000. The test has been modified by Stuber, Russman, and Proebsting, using Bayer's modification. To about 4 c.c. of neutral or faintly acid adrenaline solution is added one drop of cold saturated mercuric chloride solution acidified with 1% of $N/200$ sulphuric acid, three drops of cold saturated sulphanilic acid, and one drop of $N/10$ potassium bi-iodate solution. Upon boiling for one minute a yellowish-brown, reddish-yellow or pale yellow colour develops, increasing in intensity for several hours and then remaining constant. It is claimed that the final tint can detect adrenaline in a dilution of 1 in 100,000,000.

4. *Ferric chloride.* Ferric chloride gives a grass-green colour, immediately changing to violet and then to red upon addition of a few drops of dilute sodium hydroxide solution. Zechno and Nischo describe the following test:—One drop of ferric chloride solution is added to 1 c.c. of adrenaline solution, the concentration of the ferric chloride being varied roughly according to the amount of adrenaline present, *i. e.* 50, 5, or 0.5% for concentrations of adrenaline of 1 in 100, 1 in 1,000 or less. For a more dilute solution of adrenaline 6–10 c.c. of solution can be taken and a control test carried out with water. A red-brown or brown-yellow colour is detectable in dilutions up to 1 in 300,000. The sensitiveness is increased by sulphanilic acid.

The British Pharmacopoeia, 1932, describes the test with ferric chloride.

5. *Zamfrognini's reagent.* Thirty drops of lactic acid are added to 5 c.c. of 10% permanganate solution and diluted until

the colour disappears. This reagent detects 1 in 3 million of adrenaline.

Colour reactions have also been described with potassium ferricyanide, potassium permanganate, manganese oxides, and dichromate solutions, but they are also given by catechol and other substances containing two adjacent OH-groups. On treating a minute drop of adrenaline solution with a few drops of sodium hydroxide solution an odour resembling that of phosphoretted hydrogen is produced. The reaction is very sensitive and is characteristic of adrenaline.

The Pharmaceutical Chemistry Sub-committee of the Pharmacopoeia Commission (General Medical Council, London, 1931) recommends the ferric chloride test for adrenaline. To a neutral or faintly acid solution of adrenaline add one drop of 0.25% w/v solution of ferric chloride. An emerald-green colour develops, and, on gradual addition of 5% w/v solution of sodium bicarbonate, the colour changes first to blue and then to red.

Estimation

1. CHEMICAL

The method of Folin, Cannon and Denis depends upon the estimation of the blue colour developed in phosphotungstic acid upon reduction (*J. Biol. Chem.*, 1913, **13**, 479). Their phosphotungstic acid reagent is prepared as follows:—Boil gently for 2 hours 100 grm. of sodium tungstate, 80 c.c. of 85% phosphoric acid, and 750 c.c. of water, and make up to one litre when cold. The weighed gland is ground with sand and the mixture is rubbed in a mortar with *N* hydrochloric acid, and rinsed into a flask, 15 c.c. of acid to 2 grm. of gland being used. The mortar is washed three times with an equal volume of water. The mixture is heated to boiling, 10% sodium acetate solution is added (1/3 the volume of the hydrochloric acid used), and the boiling is continued. The whole mixture containing precipitated protein is transferred to a measuring flask (50 c.c. per grm. of gland) and made up to volume. A part of the extract is centrifuged, and 5 c.c. of the clear filtrate are measured into a 100 c.c. flask. One c.c. of 0.1% uric acid solution is measured into a similar flask, and 2 c.c. of phosphotungstic acid reagent and 20 c.c. of saturated sodium carbonate solution are added to each.

They are allowed to stand for 2–3 minutes, diluted to 100 c.c., shaken well, and compared in the colorimeter. The amount of adrenaline is calculated from the known fact that it gives a colour which is identical with, but three times as intense as, that given by uric acid. Results by this method agree well with those given by physiological assay.

Other standards have been used, *e.g.* 0.01% water-blue solution (4 c.c.), 0.01% nigrosine solution (4 c.c.), 10% $CuSO_4.5H_2O$ (10 c.c.), and 10 c.c. of hydrochloric acid (D 1.050) are mixed and made up to 100 c.c. When this solution is set at 20.6 mm. on the colorimeter, it is claimed that the colour will match that developed by 0.3 mg. of uric acid (*i. e.* the same as that given by 0.1 mg. of adrenaline), and it may be checked against the uric acid colour.

For mixtures of adrenaline with cocaine, novocaine, etc., the phosphtungstate reagent of Folin, Cannon, and Denis is not suitable, because it precipitates these bases. Either of the following methods must then be used.

Seidell's method (*J. Biol. Chem.*, *1913*, **15**, *197*).—10 c.c. of the gland extract, containing about 1 part in 50,000 of adrenaline, are shaken with 5 mg. of manganese dioxide, the mixture is filtered, and the colour of the filtrate is matched with standards prepared from mixtures made from the following solutions:—

(*a*) 2 grm. of cobaltous chloride ($CoCl_2.6H_2O$) are dissolved in water, 1 c.c. of concentrated hydrochloric acid is added, and the solution is made up to 100 c.c.

(*b*) 0.1 grm. of auric chloride in 100 c.c. of water.

Mixtures are standardised by comparison with 1/100,000 pure adrenaline solution.

Johannesohn recommends the quantitative application of the Fränckel-Allers reaction (see p. 377).

2. PHYSIOLOGICAL

The rotation of a sample of adrenaline should indicate its purity, but many samples showing rotations from $-49°$ to $-53°$ with the sodium line, correspond on physiological assay to only 64–88% of standard. It is therefore necessary to carry out a physiological estimation. The most satisfactory methods involve determination of the rise in blood pressure produced by intravenous injections of adrenaline in the anaesthetised atropinised dog, or the decapitated

cat, and are recommended by the Health Organisation of the League of Nations (E. Knaffl-Lenz, *loc. cit.*, p. 44).

The standard is a sample of pure crystalline laevo-rotatory adrenaline issued by the Health Organisation of the League of Nations.

The following method is based on one originated by H. H, Dale (see J. H. Burn, "*Methods of Biological Assay*," London. 1928, p. 46).

A cat weighing 3–4 kg. is anaesthetised with ether; one carotid artery is prepared for recording the blood pressure, and a cannula is inserted into a femoral vein. The animal is then turned into the prone position with pads under the chest and loins. A long incision is made down the midline from the top of the skull to the upper dorsal region of the spine. The muscles are separated in the midline until the spinous process of the axis (second cervical vertebra) is exposed, and held apart by means of hooks on each side. The muscles are deflected from the bone, and the spinous process of the axis is carefully nibbled away with fine bone forceps. Bleeding may be stopped with cotton wool swabs wrung out with hot saline; plasticine is useful for controlling haemorrhage from bone. The other carotid artery is then ligatured and artificial ventilation with the pump commenced.

Anaesthetisation should be as deep as possible to minimise the loss of blood. If desired, a McDowall's clamp may be applied to the vertebral arteries in the lower cervical region (*J. Physiol.*, 1930, **69**, Proc. xi). The dorsal part of the atlas and arch of the axis are now carefully nibbled away with bone forceps, exposing the spinal cord. An incision is made in the dura, a ligature on an aneurysm needle passed round the cord and tied, and a thick probe immediately thrust through the foramen magnum into the skull and the brain destroyed. After its withdrawal a tapering cone of plasticine is passed through into the brain and a small cork pushed into the foramen so as to block it tightly. The last stages of the operation from the exposure of the spinal cord should be carried out as quickly as possible; haemorrhage should be neglected until the foramen magnum has been blocked. There should be a space of about 1 cm. between the lower end of the cork and the upper end of the severed spinal cord. As soon as the brain has been pithed, the anaesthetic is turned off and artificial ventilation continued with

air alone. When all haemorrhage has ceased the vertebral clamp is removed, and the animal is placed in the supine position and prepared for recording blood pressure on the kymograph.

The object of the assay is to determine the dose which produces a rise of blood pressure exactly equal to that given by the selected dose of the standard. The standard should be dissolved in water at a concentration of 0.1% and the solution brought to an acidity of *p*H 2 by addition of hydrochloric acid. The further dilution of this and other preparations of similar concentration is usually 1 in 100, and should be made with 0.9% sodium chloride solution acidified to *p*H 2 with hydrochloric acid. A dose of the standard should be selected which produces a rise of blood pressure a few millimetres below the maximum possible rise. 0.005 mg. is commonly the most suitable dose, but it may vary between 0.002 and 0.01 mg. The injections may be commenced about one hour after the preparation of the animal, when the blood pressure has fallen to a steady low level; they should be given at regular intervals of a few minutes, that is, as soon as the pressure has returned to the basal level.

Once the standard dose has been chosen it should be given alternately with a dose of the sample under test. It is essential to repeat each dose of the latter at least once, and an assay can only be considered satisfactory when doses have been found which give rises of blood pressure just less than, equal to, and just greater than, those produced by injections of the dose of standard. The result of the assay is then accurate to within $\pm 5\%$.

The United States Pharmacopeoia (Xth Revision) recommends the use of anaesthetised atropinised dogs. The general principles of the assay are exactly the same as when the decapitated cat is used. The sample under examination is compared with a solution of standard epinephrine.

J. C. Munch and W. A. Deckert (*J. Amer. Pharm. Assoc.*, 1930, **19**, 354) found that procaine potentiated the pressor effect of adrenaline in the dog. In carrying out the test, therefore, on the solution containing procaine, a series of doses of the solution of standard adrenaline is first given to determine the sensitivity of the animal, and followed by a single injection of the solution under test. The pressor response given by the latter is then equated with that given by one of the doses of standard.

Uses and Toxicology

Adrenaline stimulates the endings of sympathetic nerves throughout the body. On intravenous injection it produces a marked rise of blood pressure together with some slowing of the heart. On subcutaneous injection it produces an intense local vaso-constriction which interferes with its absorption, so that the rise in blood pressure is less marked; the pulse rate is accelerated. Its principal use is as a local styptic, since, owing to the rise of pressure produced on intravenous injection it cannot be used in the control of deep seated haemorrhage. It finds its chief application in controlling haemorrhage from the nose, teeth, stomach or rectum. It is also frequently employed in solution with local anaesthetics since by delaying their absorption it intensifies and prolongs their action. It is of value in stimulating the heart in cases of collapse, especially when it can be injected directly into this organ. It relieves the spasms of asthma by dilating the bronchioles, and is of value in hay fever, anaphylaxis, and whooping cough. It has also been used for controlling insulin hypoglycaemia. In these cases it cannot be given by oral administration, since it is only absorbed slowly and immediately destroyed.

An over-dose produces palpitation, dilatation of the pupils, and subjective signs of fear with faintness and giddiness, tremors, tingling, pins and needles, etc.

It is contra-indicated in chloroform anaesthesia, for death can occur from stoppage of the heart owing to the onset of ventricular fibrillation.

PHARMACOPOEIAL PREPARATIONS

Adrenaline and the solution of the hydrochloride are official in the British Pharmacopoeia, 1932, U.S.P.X., D.A.B.VI. and the French Pharmacopoeia, 1920.

Adrenaline is usually employed as a solution of the hydrochloride or other salt in a concentration of 0.1%. Chloroform, chloretone, or sodium hydrogen sulphite is added as preservative, and isotonicity is ensured by the addition of sodium chloride. In the B.P. 1932 chlorbutol is added as preservative, and the solution is sterilised by heating in a closed container.

The average dose is 0.5 mg., and 1 mg. should not be exceeded in a single dose. Up to 4 mg. may be given daily. The B.P. 1932 gives a dosage range of 0.1–0.5 mg.

Adrenaline may be a constitutent of sprays, suppositories, or ointments required for local application, as in the nose or rectum. It is also frequently present in solutions used for local anaesthesia.

THE HORMONE OF THE CORTEX OF THE SUPRARENAL GLANDS

Preparation

In the method described by W. W. Swingle and J. J. Pfiffner (*Amer. J. Physiol.*, 1931, **96**, 153, 164, 180; **98**, 144) active extracts are obtained from the minced cortex or whole suprarenal gland by alternate extraction and evaporation, with aqueous alcohol, benzene, acetone and 95% alcohol as solvents. The final solution is filtered through permutite to remove adrenaline, or the material can be taken up in ether and the solution washed with sodium hydroxide solution. The alcoholic solution is diluted with water and the alcohol removed. The solution contains not more than 1 in 2 to 1 in 4 million parts of adrenaline.

According to the method of F. A. Hartman and K. A. Brownell (*Science*, 1931, **73**, 620) the minced cortex is extracted with ether in an atmosphere of carbon dioxide, and the residue after removal of the ether treated successively with alcohol of diminishing strength in the cold. After solution again in ether the residue is finally dissolved in 0.9% sodium chloride solution.

All solutions are filtered through a Seitz filter before clinical use.

Properties

The cortical hormone is soluble in organic solvents and in water. It is stable in solution in oil, alcohol, benzene and acetone, and in 0.1% benzoic acid solution. It is destroyed by boiling for two minutes and by exposure to a temperature of 80° for five minutes. It is also destroyed by cold sodium ethylate. It is not a protein.

Physiological Assay

The methods proposed are at present only qualitative or roughly quantitative. Assays by means of cats are described by Swingle and Pfiffner (*loc. cit.*), and Hartman, Brownell and W. E. Hartman (*Amer. J. Physiol.*, 1930, **95**, 670).

The criterion of activity is the maintenance in good health of animals from which both suprarenal glands have been removed in two stages, the second gland being excised about ten days after the first. Such animals usually die after about seven days. The subcutaneous or intravenous injection of a potent extract in sufficient amount is capable of prolonging life indefinitely. The amount required daily to keep the animals in good health is a rough estimate of the potency of the preparation.

Use

Aqueous extracts of the cortex of the suprarenal glands of cattle are employed in the treatment of Addison's disease, being given by subcutaneous or intravenous injection.

INSULIN

Insulin is the active constituent of the internal secretion of the pancreas, and is formed by the cells of the "islets of Langerhans." The islets lie amongst the alveoli which form the external digestive secretion, and are probably developed from the ducts of the gland. Their cells are of two types: one contains oxyphil granules insoluble in alcohol, in the other the granules are basophil and soluble in aqueous alcohol, like insulin. The islets form separate masses of cells in teleosts; in elasmobranchs the cells surround a cavity.

Insulin is the hormone which controls the metabolism of carbohydrate in the body.

Insulin is obtained commercially from the glands of the ox, sheep, or pig. It is also said to be present in most animal tissues, and substances which reduce the blood sugar have been extracted from a variety of plants.

Insulin has not yet been defined chemically, and has only recently been obtained in a crystalline form. It can therefore only be recognised by its physiological effects and assayed quantitatively by biological methods.

The unit of insulin was first defined as that amount which, when injected into a 2 kg. rabbit, caused a lowering of the blood sugar to 0.045 mg. per 100 c.c. About the same time the "clinical" unit was defined as one-third of a "rabbit" unit. Later the "rabbit" unit was halved, that is, it became the amount required per kg. to lower the blood sugar to the convulsive level, and the "clinical"

unit was increased in strength about 40%, so that these two became approximately equal. Thus, until the position was clearly defined by the Permanent Commission on Standardisation of Biological Products of the League of Nations, there were three different units bearing the approximate ratio to each other of 6:2:3. The standard at present in use is the International Standard adopted by the Health Organisation of the League of Nations (The Biological Standardisation of Insulin. *Publications of the League of Nations*, III. Health, 1926, III. 7. C.H. 398. p. 7); it is defined as containing 8 U/mg., the unit having about the same value as the revised "clinical" unit.

Crystalline insulin, which, if it is not the pure hormone itself, must be a compound containing it in a constant proportion, has an average activity of 23.3 U/mg. (There is some doubt as to whether the same result is obtained by the two general methods of insulin assay, one on mice and the other on rabbits. In general, the results from mice are somewhat higher, and those from rabbits slightly lower, than the figure given here).

Failure to use the standard in the past frequently throws doubt upon the figures given in the literature for yields of active material, but since the standard has come into general use, estimates expressed in international units of activity are comparable.

Preparation

The following outline of the preparation of insulin is taken from a paper by F. H. Carr (*Chem. & Ind.*, 1926, **45**, 750):—

1. Pancreas glands, obtained as soon as possible after the death of the animal, are ground and minced at a low temperature.

2. The material is rendered acid to *p*H 2.5, or alkaline to *p*H 7, in order to set free the insulin from the tissue on which it is adsorbed.

3. The mixture is extracted with 65–70% alcohol, a concentration in which the proteolytic enzymes are insoluble.

4. The extract is clarified by cooling to -5° and filtering or centrifuging.

5. The extract is concentrated at a low temperature to one-tenth of its volume and filtered after extracting the fat.

6. The proteins are precipitated with ammonium sulphate or with absolute alcohol at *p*H 4.5.

7. The protein mixture is precipitated by adjusting the aqueous solution to *p*H 5.2.

8. The precipitate is converted to picrate and regenerated as a hydrochloride by Dudley's method (*vide infra*).

The method of Dickens and Dodds avoids the use of large quantities of alcohol (E. C. Dodds and F. Dickens, *Lancet*, 1924, **1**, 330; *Brit. J. Exp. Path.*, 1924, **5**, 115). One kg. of pancreas is minced and mixed thoroughly with 45 grm. of dry powdered picric acid. After repeating the mincing twice, the material is extracted twice with 500 c.c. of dry acetone and twice with 500 c.c. of 70% acetone. The liquid is separated by filtration under pressure. The acetone is evaporated from the extract by distillation *in vacuo*, and an equal volume of saturated picric acid is added to precipitate the picrate. This is treated with dry ether to remove fat and excess of picric acid.

The picrate is converted into hydrochloride by Dudley's method (H. W. Dudley and W. W. Starling, *Biochem. J.*, 1924, **18**, 147). It is dissolved in 0.75 *N* hydrochloric acid 75% alcohol (10–20 c.c. per grm. of picrate) and the solution centrifuged. The hydrochloride is precipitated from the resulting clear solution by addition of 10–20 volumes of acetone. It is washed on the filter with acetone and ether and dried in vacuo.

Other methods of purification include the method of isoelectric precipitation (M. Somogyi, E. A. Doisy, and P. A. Schaffer, *J. Biol. Chem.*, 1924, **60**, 31), adsorption on to benzoic acid, the benzoic acid being subsequently removed by ether (P. J. Moloney and D. M. Findlay, *J. Biol. Chem.*, 1923, **57**, 359), or adsorption on to charcoal and elution by washing with acid or phenol (E. Dingemanse, *Arch. exp. Path. Pharm.*, 1928, **128**, 44).

If it is desired to obtain free insulin, the hydrochloride must be dissolved in slightly acid water and the requisite amount of ammonia added until maximum precipitation occurs at about *p*H 5.0. At a more alkaline reaction insulin, being an amphoteric substance, redissolves. In general, it is more convenient to handle insulin as the hydrochloride, which is more easily dissolved than iso-electric insulin, and does not exhibit the same tendency to change to a gummy form. The precipitate of hydrochloride or of the base itself is washed with alcohol, acetone and ether, and dried in a desiccator.

CRYSTALLINE INSULIN

Neither of the two methods in use for the preparation of crystalline insulin can be regarded as satisfactory, as the yield of crystals in both

cases is very small unless the treatment is repeated a large number of times, and there is little increase in potency. Several of the brands of insulin now on the market are already of activity of the order of 20 U/mg. before crystallisation is attempted.

1. **Saponin Method** (C. R. Harington and D. A. Scott, *Biochem. J.*, 1929, **23**, 384).—In the original method small batches were treated separately because the ease of crystallisation depends largely on the ratio of the volume of the solution to the area of the glass surface with which it is in contact. 1 c.c. of 6% saponin solution is added to 5 c.c. of a solution in 2% acetic acid of 20 mg. of insulin, the potency of which is 14 or more units per mg. 1.6 c.c. of 0.85% ammonia is added to the mixture, which is warmed to 35° and kept at that temperature for 30 minutes. The precipitate is removed by centrifuging, and the mother liquor is adjusted to *p*H 5.6 by immediate addition of 1.7 c.c. of ammonia, and transferred to a 15 c.c. centrifuge tube. On standing, and scratching with a glass rod a turbidity develops and a precipitate separates which is largely microcrystalline. The precipitates obtained from four or five tubes are mixed in solution in 2.5 c.c. of 10% acetic acid and 10 c.c. of water. 2.6 c.c. of 6% saponin are added, and the reaction adjusted to *p*H 5.6 by addition of 8.25 c.c. of 0.85% ammonia. The precipitate that forms is more definitely crystalline than the first. The mother liquor may be saturated with butyl alcohol by which means a further crop of crystals is obtained. Finally, the whole of the partially crystalline precipitate is dissolved in 0.4 c.c. of 0.1 *N* hydrochloric acid and 4 c.c. of water, and the solution is poured into a mixture of 16 c.c. of phosphate buffer *p*H 7.0 (W. M. Clark, *The Determination of Hydrogen Ions*, 2nd Ed., 1923), and 0.8 c.c. of 0.1 *N* sodium hydroxide solution. The solution is warmed to about 55° and treated with 1 c.c. of 0.1 *N* hydrochloric acid to bring the reaction to *p*H 5.6. Within a few hours a wholly crystalline precipitate separates. The crystallisation from phosphate buffer can be repeated once or twice, by which means the crystals are enlarged in size. The yield obtained is only 5–15%, but the treatment can be applied to the solid recovered from the mother liquors. Crystalline insulin can also be obtained without the preliminary saponin treatment by repeated solution and precipitation in the phosphate buffer. After the final crystallisation the product is washed twice with distilled water and dried *in vacuo* over sulphuric

acid. Scott has shown that insulin from different sources varies greatly in the ease with which it responds to this treatment, cod insulin being more easily obtained in crystalline form than that from the pig or the sheep (*J. Biol. Chem.*, 1931, **92,** 281).

The kind of saponin used has a great influence upon the yield; for the purpose of selection the haemolytic index appears to be of some significance, while the frothing power has no connection with its efficiency in promoting crystallisation.

2. Abel's Method (*J. Pharm. Exp. Ther.*, 1927, **31,** 65).—Two grm. of insulin hydrochloride (activity about 13 U/mg.) are dissolved in 20 c.c. of 10% acetic acid, and 80 c.c. of a solution of brucine are added (1 grm. of brucine in 18 c.c. of $N/6$ acetic acid). If a small dark coloured precipitate separates, it is removed, and 40 c.c. of 13.5% pyridine are added to the filtrate slowly with stirring. A considerable precipitate forms, which is removed on the centrifuge, care being taken that the temperature of the contents of the tubes does not exceed 37°. To the filtrate 40 c.c. of 0.65% ammonia are added slowly, with constant shaking, and if a precipitate develops, it is removed on the centrifuge (1st ammonia ppt.). As the *p*H of the solution at this stage is very near the iso-electric point of insulin (5.5–5.6) the supernatant fluid may remain cloudy. It is poured into a flask and set aside, unless a flocculent precipitate appears at once, when it is advisable to remove it before setting the flask aside for crystallisation (2nd ammonia ppt.). After standing for about 12 hours crystals are found upon the sides of the flask. They are washed with 0.65% ammonia, then repeatedly with cold water and finally with absolute alcohol, by which all traces of brucine are removed. The yield is about 14%.

The ammonia precipitates consist largely of crystalline insulin. They are dissolved in 10% acetic acid and treated as before, omitting the brucine and pyridine treatments. The pyridine precipitate may be treated similarly.

The mother liquors are combined and evaporated in a current of air, and the moist residues are treated repeatedly, first with absolute alcohol containing a very little acetic acid, then with absolute alcohol alone until only a trace of brucine remains in the dry residue. The residue may be treated as above and a further small crop of crystals obtained from it. The total yield of crystalline insulin is about 26%. In order to get the maximum yield it is advisable to

let the flasks stand for several days and to use rather dilute solutions Crystals obtained by this method and by the method of Haringtoı and Scott have an activity of 23.3 U/mg.

Analysis of Crystalline Insulin

Many elementary analyses have been carried out upon insulin The figures given below are those for crystalline samples. Othe results published for "insulin" were actually obtained on sample of such low activity that some contained not more than about 8% o the active material, reckoned as crystalline insulin.

Cystine, tyrosine, arginine, histidine, and leucine have beer isolated from the hydrolysate of crystalline insulin. Lysine i: probably present also, and there is some evidence of a sulphur com pound other than crystine.

TABLE II

	Harington and Scott, (*Biochem. J.*, 1929, **23**, 384)		Abel *et al.* (*J. Pharm Exp. Ther.*, 1927, **31**, 65)	Calculated for $C_{45}H_{69}O_{14}N_{11}S$, $3H_2O$ or $C_{45}H_{75}O_{17}N_{11}S$ (Abel)
C	49 61	49.11	49.91	50 19
H	6 81	6.95	7.16	7 22
O	.....	.....	25.58	25 28
N	14.49	14.03	14.41	14.32
S	3.11	.....	2.94	2.98
			100.00	99.99
			H_2O (5.35)	(5.03)

From nitrogen determinations by the method of Van Slyke, the following quantities of the different amino acids have been calculatec to be present:—

Arginine.. 3.22%
Cystine.. 8.16%
Histidine.. 4.40%
Lysine.. 2.26%

(V. du Vigneaud, H. Jensen, and O. Wintersteiner, *J. Pharm. Exp Ther.*, 1927, **32**, 367, 387.)

Properties

Insulin, as it is met with in commerce, is an amorphous white powder. The crystalline form is at present very seldom encountered

owing to the difficulties of its preparation, and to the fact that amorphous material of nearly equal physiological activity is readily obtained.

Insulin is soluble in acids or alkalies, except at its iso-electric point *p*H 5.5. It is also precipitated by 3.3% hydrochloric acid, but dissolves again on adding a further excess. It is soluble in aqueous alcohol and in aqueous acetone, but is insoluble in absolute alcohol high concentrations of acetone, in ether, chloroform, benzene, and other inert organic reagents. Insulin is soluble in phenol, in dry liquid acetamide, and in liquid ammonia, but after it has been inactivated by cysteine or glutathione it loses its solubility in acetamide. It is insoluble in pyridine.

Abel (*J. Pharm. Exp. Ther.*, 1927, **31**, 65) has given the melting point of crystalline insulin as 233°, and has found a specific rotation of −40° for a 0.5% solution in 0.002 *N* hydrochloric acid, but there was a great deal of variation in the rotation of different samples, upon which the concentration and nature of the solvent exerted a marked influence, for example—

Sample 1, in 10% acetic acid gave $[\alpha]_D$ −22°
1% in *N*/6 acetic acid $[\alpha]_D$ −19°
on recrystallisation 1% in *N*/6 acetic acid $[\alpha]_D$ −22°

Sample 2, in *N*/6 acetic acid $[\alpha]_D$ −30°
in 0.01 *N* acetic acid $[\alpha]_D$ −17°

These rotations remained unchanged for days.

Sample 3, in 1.3% liquid ammonia gave $[\alpha]_D$ −48°. Part of the solution after standing for 3 days showed an increased rotation to −63°, and after a further 9 days, it went opalescent and yellow, and gave a rotation of −57°. It was not possible to recover crystalline insulin from it. When the original ammonia solution was immediately acidified by means of two drops of concentrated hydrochloric acid, the rotation changed to −32°.

The spectrum of crystalline and amorphous insulin of high activity shows an absorption with a maximum at 2,750 Å, but it remains unchanged when the physiological activity is lost by action of alkali or by heating the acid coagulum. Loss of activity by methylation is accompanied by a change in absorption (W. Kuhn, H. Eyer and K. Freudenberg, *Z. physiol. Chem.*, 1931, **202**, 97).

Insulin crystals are well defined and usually less than 0.1 mm. in diameter; they fall into two general groups:—(1) crystals in the

rhombohedral class with well defined total refraction. They present the appearance of hexagons owing to the rhombohedra standing on one corner. They have a refractive index of 1.58. (2) crystals with no definite refraction, the faces being diamond shaped.

In the absence of air insulin is stable to X-rays, radium, and ultra-violet irradiation, but in the presence of air, radium and ultra-violet light destroy it. Insulin is inactivated partially or completely by formaldehyde, by acetylation, by the combined action of alcohol and acids, by the action of heat on the alkaline solution, by pepsin and trypsin, glutathione, cysteine, and a variety of other agents. The inactivation may be reversible or irreversible.

Recorded values for the activity of the purest known samples are as follows:—

100, 125	Units per mg.	(Abel)
90	" " "	(Abel, Geiling, Bell and Wintersteiner)
54	" " "	(Du Vigneaud, Geiling and Eddy)
45	" " "	(Jensen and Geiling)
125, 167	" " "	(Dingemanse)
23, 26	" " "	(Freudenberg and Dirscherl)
23.3	" " "	(Culhane, Marks, Scott and Trevan)
27	" " "	(Scott)
100	" " "	(Dirscherl)
20.1	" " "	(by rabbit test (Culhane and Greenwood))
24.5	" " "	(by mouse test (" " "))

All except the last six (the most recent) were obtained by the old method of direct calculation from the degree of hypoglycaemia produced in rabbits without reference to any standard.

The following properties are taken chiefly from the observations of Abel and his co-workers:—

When insulin is dried at 105–120° in nitrogen under reduced pressure, water is given off varying in amount between 4.60 and 6.34%, usually 5.35 ± 0.3%.

Insulin gives the biuret, Pauly, Millon, and ninhydrin reactions. Recrystallised insulin does not give the tryptophane reactions of Voisonet, Hopkins and Cole, or Acree.

It is readily attacked by bromine water with the production of one or more alcohol soluble crystalline substances. Upon boiling for a short time with aqueous sodium hydroxide in a concentration less than 10%, a crystalline degradation product is obtained, which

is soluble in alcohol, and free from sulphur, but gives the biuret and Pauly reactions.

Reduction of insulin causes its inactivation, and the liberation of 0.06% of ammonia.

Reversible Inactivation of Insulin

1. By acetic anhydride. When insulin is precipitated from methyl alcohol by benzene, and the suspension is treated with pyridine and acetic anhydride at 0° for 3 hours, an acetyl derivative is obtained which is almost inactive. A product containing about one-fifth to one-third of the activity of the original insulin may be recovered by the action of 0.03 *N* sodium hydroxide at 1–3° for 24 hours (K. Freudenberg and W. Dirscherl, *Z. physiol. Chem.*, 1928, **175**, 1). Upon a second acetylation and hydrolysis only 20% of the activity is lost. According to Jensen and Geiling the reaction is effected much more readily (*J. Pharm. Exp. Ther.*, 1928, **33**, 511). Slight differences between their observations and those of Freudenberg and Dirscherl they ascribed to the fact that the latter used less pure insulin for the inactivation.

2. By the combined action of alcohol and acid. Insulin can be completely inactivated by treatment with mixtures of strong acids and primary or secondary alcohols, and the potency recovered by treatment with dilute sodium hydroxide (F. H. Carr, K. Culhane, A. T. Fuller and S. W. F. Underhill, *Biochem. J.*, 1929, **23**, 1010). When insulin stands in solution at room temperature in a mixture of 3 parts of anhydrous ethyl alcohol and 1 part of 3 *N* hydrochloric acid, it is almost completely inactivated at the end of 30 hours. At the same time the *p*H of maximum precipitation changes from 5.0 to 6.0. By adding an equal volume of 0.0842 *N* sodium hydroxide to a 1% solution of inactivated insulin and keeping the mixture at 0° for 17 hours, the activity can be restored, the reversal of the *p*H change being also effected. For this reaction the concentration of the alkali is of the greatest importance. The work was repeated by Charles and Scott using crystalline insulin (*J. Biol. Chem.*, 1931, **92**, 289). They confirmed the inactivation, and obtained a 60% recovery upon treatment with a rather lower concentration of sodium hydroxide; crystals prepared from the reactivated insulin had the same potency as the original material.

3. The slow inactivation of insulin by means of formaldehyde is partly reversible under the action of very dilute hydrochloric acid.

4. Treatment with diazomethane inactivates insulin, but 30–40% of the original activity is restored by regeneration. The reaction with diazomethane suggests that the esterifiable-COOH group is free and does not occur as a lactone.

Irreversible Inactivation of Insulin

The inactivation of insulin by pepsin at *p*H 2 is accompanied by an increase in amino nitrogen of 0.4%, by trypsin-kinase at *p*H 8 of over 4%, and by papain at *p*H 3.6 or 6, of 1.4% (K. Freudenberg, W. Dirscherl, H. Eichel and E. Weiss, *Z. physiol. Chem.*, 1931, **202,** 159).

Insulin is irreversibly inactivated by cysteine and glutathione. Compounds of insulin with benzaldehyde and benzoyl chloride are inactive, and the activity cannot be regained. Insulin is inactivated by the prolonged action of cyanide and nitrite in concentrations greater than 0.05 *N*, and by diazobenzene sulphonic acid in faintly alkaline solution. Iodine destroys the activity but the amount taken up bears no relation to the unit content. Benzoylation destroys the activity, and Blatherwick and co-workers could not restore it by hydrolysis (N. R. Blatherwick, F. Bischoff, L. C. Maxwell, J. Berger and J. Sahyun, *J. Biol. Chem.*, 1927, **72,** 57). Insulin is destroyed by carbon disulphide and by reducing agents.

Insulin is inactivated by treatment with *N*/30 sodium hydroxide solution at 30° for 3 hours, and 0.14–0.17% ammonia is liberated. It is more resistant to acid, but heating for one hour with *N*/10 hydrochloric acid causes 0.5% of ammonia to be split off, the activity remaining unaltered. 0.5 *N* hydrochloric acid in 30 days at 32° causes the removal of 1% of ammonia. The fact that some ammonia can be split off by acid without loss of activity indicates that it comes from a different part of the molecule from that from which it is liberated by alkali. For further work on the inactivation of insulin and its bearing on the chemical constitution, the original paper by Freudenberg (*loc. cit.*, p. 128) should be consulted.

Action of Heat on Insulin

Insulin can be precipitated from acid solution by heat. If it is heated in 0.1 *N* hydrochloric acid on the water-bath for one hour,

and the precipitate removed on the centrifuge, it is still readily soluble in alkali. The filtrate is inactive, but the precipitate, when dissolved by means of sodium hydroxide remains in solution upon acidification and retains four-fifths of its former activity. Crystalline insulin precipitated by heating with 0.1 *N* hydrochloric acid *cannot be re-converted to crystalline form.*

Dingemanse (*Biochem. Z.*, 1925, **163**, 422) obtained the following results for the action of heat on acid solutions of insulin:—

In *N*/100 HCl boiling for 1 hour had little effect.
In *N*/100 HCl boiling for 2 hours caused loss of half the activity.
In *N*/10 HCl boiling for 2 hours greatly reduced the activity.
In 25% HCl boiling for 2 hours almost completely destroyed the activity.

Solid insulin can be kept unchanged indefinitely, and even the solution at *p*H 3.6 deteriorates very slowly. Indeed, it is highly probable that there is no deterioration at all in years, of solutions of insulin of the highest purity, provided that bacterial action is excluded and that they are kept cold. A solution of crystalline insulin prepared 3 years ago has recently been re-tested by the authors and found to have retained its full potency.

H. Krogh and A. M. Hemingsen (*Biochem. J.*, 1928, **22**, 1231), working on solutions of commercial insulin, obtained the following values by experiment and calculation for the rate of inactivation of insulin at different temperatures.—

Temperature	Time taken for activity to fall		
	5%	10%	50%
40°	½ month	1 month	½ year
30°	2 months	4 months	2 years
20°	9 months	1½ years	10 years
10°	4½ years	8½ years	86 years
0°	29 years	58 years	377 years

Comments by various authors on the stability of insulin in acid solution are somewhat misleading for the analyst, for while it is much less readily inactivated by heat when the solution is on the acid side of the isoelectric point, insulin must be heated with great caution. Thus in an unpublished experiment a solution at *p*H 3.6 showed the following losses:—

Heated for 5 hours at 100°	loss more than 50%	
Heated for 1 hour at 100°	"	50–75%
Heated for 5 hours at 50°	"	12.5%
Heated for 61 days at 37.5°	"	27%

Insulin solution can safely be kept for clinical use for at least two years at room temperature, or in the cold indefinitely.

Sulphur in Insulin

From the time of its discovery there has been a tendency to associate the activity of insulin with its sulphur content, and it is a fact that du Vigneaud found that the fraction of insulin purified by Abel and Geiling's method had a high cystine content, and he has even gone so far as to attempt to base a chemical method of assay upon this observation. Insulin itself does not react with Sullivan's reagent for cystine, but the product of its hydrolysis gives a positive reaction. It is therefore probable that cystine is present in peptide linkage and the sulphur as a disulphide. Heated with 0.1 *N* sodium carbonate solution, most of the sulphur in insulin is eliminated as sulphide, but the hydrolysed product does not yield sulphur so readily.

A portion of the sulphur which is labile to sodium hydroxide can be oxidised by dilute nitrate without loss of activity; conversely, the activity can be destroyed by iodine without affecting the sulphur labile to sodium carbonate (N. R. Blatherwick, *et al., loc. cit.*). It has been found that small amounts of ammonia are given off from crystalline insulin with the "labile" sulphur (about 3% of the total nitrogen). The sulphur and nitrogen set free are nearly proportional to their atomic weights. The significance of this is doubtful, and it does not appear that there is any direct connection between the sulphur content and the activity.

DERIVATIVES OF INSULIN

Acetyl Insulin.—100 mg. of finely powdered crystalline insulin are thoroughly mixed with 10 c.c. of freshly prepared acetic anhydride, and the mixture kept on ice for 15 hours. Upon addition of water acetyl insulin is precipitated and is filtered off and washed with water, alcohol, and ether.

Properties.—Acetyl insulin is insoluble in water, alcohol, and acid, but soluble in alkali and in *M*/15 disodium hydrogen phosphate. The physiological activity is about one-fifth of that of the insulin

from which it is prepared. It is very readily hydrolysed, to some extent even by $N/100$ alkali.

Other derivatives of insulin have been prepared by the action of various reagents on insulin solutions, but, for the most part, they have not been isolated, nor have they been described at all, except in so far as they have suffered any change in physiological activity.

Estimation of Insulin

There are no chemical tests for insulin, and the final identification of it depends upon physiological methods.

The test proposed by Wyss based on the inhibition of the oxidation of polyhydric phenols by hydrogen peroxide is given equally by other protein solutions.

The assay of the gland is best carried out as follows:—Ten grams of gland in thin slices are weighed into 15 c.c. of alcoholic sulphuric acid (3 vols. of 10 N sulphuric acid to 50 vols. of alcohol). The mixture is ground with sand and filtered through paper after 3 hours. The residue is re-extracted overnight with 15 c.c. of alcoholic sulphuric acid diluted to 70% alcohol strength with water. The extract is cooled, filtered, and concentrated to low bulk in an open dish at room temperature in a current of air. The residue is transferred quantitatively to a centrifuge tube, treated with chloroform and centrifuged. The clear aqueous layer is removed, and the insoluble fat is washed twice with very dilute acid. The combined aqueous solution and washings are adjusted to *p*H 3.5–4.0 with 20% ammonia, diluted, and injected.

Up to 4 units may be extracted per grm. of gland. For the purpose of assay it is convenient to dilute an aliquot portion of the extract on the basis of a yield of 2 units per grm. for a preliminary test. Test solutions should be kept cold, and a small amount of cresol (not more than 0.3%) may be added as a further precaution against bacterial action.

The weight of the fresh gland is, in the ox, about 300 grm., and in the sheep, 150 grm.; the pig's gland is slightly smaller than the sheep's.

PHYSIOLOGICAL ASSAY

Methods of physiological assay are based on the reduction of the blood sugar produced by an injection of insulin. Either this

reduction is measured directly, or its effect on the animal, namely the appearance of convulsions, is taken as the criterion. In our experience the method using rabbits described by Marks, the "cross test" (*The Biological Standardisation of Insulin. Publications of the League of Nations*, III. Health, 1926, III. 7. C. H. 398, p. 57) or that using mice described by Trevan and Boock (*ibid.*, p. 47) are the most useful. For all accurate assays it is essential to employ a method permitting the direct comparison of the sample with the standard of reference.

Standard.—The International Standard adopted by the Health Organisation of the League of Nations (*ibid.*, p. 7) at present in use contains 8 U/mg. It is dissolved at about 20 U per c.c. in acid water (*p*H 4) containing 0.3% cresol.

Qualitative Tests.—As a preliminary to a more accurate assay, time and rabbits can be saved if one has some knowledge of the hypoglycaemic potency of the sample to be tested. Two or three rabbits are starved for 22 hours and their resting blood sugars then determined. A dose of the sample is injected subcutaneously and the blood sugar followed hourly until the minimum is reached. 0.25 U per kg. usually gives a minimum at the first hour, 0.5 U one at the second, and 0.75 U one at the third hour. By noting the position of the minimum it can thus be seen whether the dose given is of the order of half a unit, or greater or less, information which is invaluable for the selection of a suitable dose for the quantitative estimation.

Quantitative Assay. 1. *The Rabbit Method.*—It is essential to use carefully selected animals, and to keep the stock under as uniform conditions as possible. Young healthy rabbits of 1600–3000 grams in weight with large ears, should be kept at a constant environmental temperature and on a constant diet, *e. g.* oats, hay, and cabbage. As an aid to their selection all animals should receive a subcutaneous injection of 0.5 U/Kg. of the standard preparation, and any which develop convulsions should be discarded. It is also advisable to discard them after they have been in use for more than two or three months, if they habitually give figures for the percentage blood sugar reduction (as defined below) of less than 15% or more than 45%, and if at any time they have received doses varying greatly from 0.5 U/Kg. Further details on the selection of rabbits are given by K. Culhane (*Quart. J. Pharm.*, 1928, **1**, 517).

The estimation is performed in two parts, the first on one day, and the second three or four days later.

First Day. An even number of rabbits (not less than ten) is deprived of food for 22 hours before the test. Before injection they are weighed and divided into two groups, such that both the total weights and the distribution of individual weights in the two groups are as far as possible the same. A sample of blood is withdrawn from the ear vein of each rabbit, 2–5 mg. of potassium oxalate being added to each c.c. to prevent clotting, and the percentage of blood sugar is determined. For large numbers of rabbits the Folin-Wu method for the estimation of blood sugar is simple and rapid, and allows one observer to carry out a large number of estimations in one day. It is essential, however, to correct for the error of the colorimeter by constructing a standard curve relating the reading to the amount of sugar present when the standard is set at 20 mm. The use of this curve considerably increases the accuracy of the method, and is quicker than other means of correction that have been proposed. Such a curve can be constructed from the

TABLE III

Column 1. Colorimeter readings when the standard is set at 20 mm.

Column 3. Actual values for the blood sugar (mg. per 100 c.c.) when the standard solution contains 0.2 mg. of glucose and 2 c.c. of the blood filtrate are taken (equivalent to 0.2 c.c. blood) (O. Folin and H. Wu, *J. Biol. Chem.*, 1920, **41**, 367). (The calculated values for the blood sugar are given in column 2).

Colorimeter reading d mm.	Blood sugar in mg. per 100 c.c. blood: Calculated from formula: Blood sugar $= \frac{20}{d} \times 100$	Blood sugar in mg. per 100 c.c. blood: Experimental
15	133	127
16	125	120
17	118	115
18	111	109
19	105	105
20	100	100
21	95	96
22	91	93
23	87	90
24	83	86
25	80	83
26	77	81
27	74	78
28	71	75

figures given in the table. An accuracy within ± 2–3 mg. glucose per 100 c.c. of blood is attained. Alternatively, the Hagedorn-Jensen method for blood sugar determination which requires no special apparatus, gives reliable results.

Each rabbit in the first group is given a subcutaneous injection of 0.25 c.c. per kg. body weight of a solution of the standard preparation containing 2 units per c.c., and each animal of the second group, 0.25 c.c. per kg. of a solution of the sample under examination expected to be equivalent. Samples of blood are withdrawn hourly for five hours after the injection, and the average blood sugar is determined. It is convenient to pool the five samples from one rabbit rather than to carry out five separate estimations; 0.2 c.c. of blood is therefore delivered into a test tube containing 8 c.c. of $N/12$ sulphuric acid at each sampling and the tube is shaken thoroughly. After addition of the last sample 1 c.c. of a 10% solution of sodium tungstate is added, slowly with shaking, and the estimation of the sugar carried out on the filtrate in the usual way. No glycolysis occurs over the five hours of the experiment.

The "percentage blood sugar reduction," that is, the difference between the initial blood sugar and the average blood sugar for the five hours, expressed as a percentage of the initial figure, is then calculated for each of the rabbits.

Second Day. Three or four days later the test is repeated, but on this occasion the animals which were given the standard preparation on the first day now receive the dose of the sample, whilst those which were given the sample now receive the dose of the standard. The percentage blood sugar reductions are determined as before.

The sum of the percentage blood sugar reductions of the animals when given the dose of sample expressed as a percentage of the corresponding sum when given the dose of standard represents the percentage activity of the unknown solution in terms of the solution of the standard.

While the total percentage reduction is roughly proportional to the dose within certain limits, as in all physiological assays it is only possible to obtain an accurate determination when the dose of the sample produces the same effect as the dose of the standard. In this case the assay may be considered accurate to within ± 10%; frequently a greater accuracy is attained. When the result of the estimation shows that the doses given differ by more than 10%, it

must be repeated using a dose of the sample per kg. body weight calculated to contain 0.5 U.

Success in the method depends chiefly on a proper selection of rabbits and of the dose given to them, and upon a rigid adherence to the exact times for taking blood samples.

The B.P. 1932 describes this method for the biological assay of insulin, using 10 or 12 rabbits.

2. *The Mouse Method.*—An inbred stock should be employed maintained at a constant environmental temperature and on a constant diet, such as brown bread soaked in water, and mixed with canary, millet, hemp, or linseed in rotation, cod-liver oil and chopped cabbage being added once, and dried cheese twice, a week. All mice are starved for the 18 hours preceding the test.

At least sixty mice, weighing between 16 and 22 grm. each, are divided into two groups as uniformly as possible. Each mouse of the first group is then given a subcutaneous injection of 0.1 c.c. of the standard solution (0.2 U per c.c. in water acidified to *p*H 4.0), and each of the second group a dose of the sample expected to be equivalent. Immediately after injection the animals are placed in small ventilated boxes immersed in a specially constructed thermostat at a constant temperature of 37.5°. Five mice are put into each box, injections being made in groups of five, standard and sample, in alternate order. The number of convulsions occurring during the ensuing 1½ hours is noted, and the percentage on standard and sample is determined. Animals developing convulsions are immediately given a subcutaneous injection of 0.2 c.c. of 20% glucose solution. They suffer no ill effects and can be used again in subsequent tests.

The result of the assay is obtained from a curve relating the dose given to the percentage developing convulsions. Such a curve is constructed by injecting the standard preparation in varying doses into a large number of mice, and may be kept as a standard of reference.

In calculating the result, the dose of standard corresponding with the observed convulsions on the standard is read off on the curve. The ratio between this and the actual unit value of the dose injected gives a factor for the day variation. Next is read from the curve the dose of standard that would correspond to the number of convulsions on the sample, and the result is divided by the day variation factor.

Thus if x and y are the percentage numbers of convulsions observed on the sample and standard respectively, and from the curve, B is the "characteristic" dose of standard to give $y\%$ convulsions, then the ratio B/0.02 gives the Day Variation Factor, 0.02 U being the actual dose of standard given.

If A is the dose from the curve corresponding to the $x\%$ convulsions obtained on the sample, the potency is then given by $A \times 0.02/B$, for the number of units contained in the 0.1 c.c. injected.

The potency of the original solution is calculated from this result by reference to the dilution made.

With the use of such a "characteristic" curve, and thirty mice on the standard preparation and on the sample, the error of the result may be considered to be $\pm 10\%$, provided that the number of convulsions on the standard is approximately equal to that on the sample. If this is not the case, the determination should be repeated, using doses of standard and sample estimated to produce an equal number of convulsions in each group of mice used. The result shows the greatest accuracy when the number of convulsions falls on the straight part of the curve (30–50%). Tests in which a very high or very low value is obtained should be repeated after adjustment of the dose.

Most commercial samples of insulin consist of slightly acid solutions of the hydrochloride in presence of a very small percentage of tricresol or other phenolic preservative. To some are added sodium chloride or glucose to bring the concentration of the solution up to or near the isotonic strength. A useful scheme for the examination of such a solution is as follows:

1. A rough physiological test should be carried out to ascertain that the solution actually contains insulin and to show approximately the order of its activity. With a fortunate choice of two different doses a good indication can be obtained from four rabbits. This should be followed by a "cross-test" on twenty rabbits.

2. A nitrogen estimation by the micro Kjeldahl method gives the amount of nitrogen per unit; samples of good commercial insulin have been found to contain about 0.007 mg. nitrogen per unit. If the nitrogen is greatly in excess of this figure, the presence of a high content of inert protein is indicated.

3. By evaporation at a temperature not exceeding 100° the total solids can be estimated. If this figure is greatly in excess of the

quantity of insulin calculated from the nitrogen content, salt or glucose is probably present.

4. The *p*H of the solution should be determined and titration of the free acid will give the total acid together with the minute amount liberated from the insulin hydrochloride.

5. The amount of sodium chloride present (if any) is determined by subtracting the figure calculated from the titration of the acid from the total chloride estimated after the complete oxidation of organic matter. This assumes the presence of hydrochloric acid. If some other acid has been found, an estimation of the total chlorides will give directly the figure for the added sodium chloride.

6. Glucose can be estimated by Folin and Wu's procedure for blood sugar.

Uses and Toxicology

Insulin is a specific in the treatment of diabetes mellitus which is due to the deficient secretion of insulin in the body. It restores the condition of the patient to that of a normal individual. Its action is shown by reduction of the blood sugar to normal limits and by improvement in the oxidation of fat following the increased utilisation of carbohydrate induced by it; the excretion of glucose and acetoacetic acid in the urine is thereby reduced or abolished. Owing to its effect on carbohydrate metabolism it has been used also in a variety of non-diabetic conditions in which it is desired to stimulate appetite and the oxidation of carbohydrates. Thus it has been employed in conditions of acidosis such as surgical shock, vomiting, etc., and to improve nutrition in conditions of emaciation, etc.

A toxic effect is observed if the blood sugar is reduced by insulin below a certain value. The onset of symptoms may be preceded by a sensation of hunger: the patient becomes uneasy and nervous, and suffers from weakness and loss of emotional control; palpitation, sweating, and pallor or flushing may occur. Unless the condition is treated, acute mental distress, incoordination, disorientation, and delusions are observed, with loss of deep reflexes, and finally delirium and coma with death. The condition is at once improved by the administration of glucose, or, if not too far advanced, by the injection of adrenaline or pituitary posterior lobe extract.

PHARMACOPOEIAL PREPARATIONS

In Great Britain insulin is scheduled by the Therapeutic Substances Regulations 1931 (Therapeutic Substances Act, 1925) (Statutory Rules and Orders 1931, No. 633). Insulin is issued only for administration by injection, either in sterile aqueous solution at a *p*H between 3 and 4, or in sterile tablets suitable for solution in water, or as a sterile suspension in oil. The potency is expressed in terms of the International Standard unit of activity. The date of manufacture should be specified on the label. In Germany, the German Insulin Committee control the testing and manufacture, and in Canada and the United States the Governors of the University of Toronto.

Solutions of insulin and tablets as described above are official in the British Pharmacopoeia 1932. The solution is sterilised by filtration and should contain an antiseptic. The potency is determined by a biological test. The dosage is given as 5–100 units by subcutaneous injection. Preparations are also described in *New and Non-official Remedies* (Amer. Med. Assoc., 1931, Chicago, p. 195).

THE HORMONES OF THE PITUITARY GLAND

The pituitary gland is a small body connected with the base of the brain by a stalk and lying in a bony fossa in the floor of the cranium. It consists of two lobes; the pars anterior, forming the anterior lobe, is separated by a cleft from the pars intermedia and the pars nervosa, which together form the posterior lobe. In addition, some granular cells associated with the upper surface of the pars anterior and extending along the stalk over the surface of the floor of the third ventricle, are described as the pars tuberalis. The development and functions of the different parts are entirely different. The partes anterior, intermedia, and tuberalis are developed from an up-growth of the buccal ectoderm, the pars nervosa from a down-growth of the neural ectoderm of the floor of the third ventricle with which it remains connected by the stalk.

The largest lobe is the anterior; it partly surrounds the pars nervosa and accounts for about 2/3 to 3/4 of the weight of the gland. It is glandular in structure and composed of a solid mass of cells well provided with capillaries. The cells contain either clear or granular cytoplasm; the granules stain with chromic acid and either

acid or basic dyes, but only one type is found in each cell. The pars intermedia, accounting for only a small proportion of the weight of the gland, closely invests the pars nervosa; it consists of small cells containing faint basophil granules. They are arranged round small vesicles containing colloid. The cells of the pars tuberalis are somewhat similar in type.

The pars nervosa, which accounts for about one-fifth of the weight of the gland, consists of neuroglial fibres and cells and contains a dark brownish-yellow pigment.

The gland is found in all vertebrates and secretes a number of hormones which are ill-defined chemically but have definite physiological actions. Only those which have been obtained in gland extracts and are likely to be of therapeutic importance can be mentioned here.

THE HORMONES OF THE POSTERIOR LOBE

Synonyms. Pituitary (posterior lobe) extract, oxytocic (pressor, antidiuretic) principle of pituitary posterior lobe, solution of pituitary, liquor pituitarii.

The posterior lobe secretes a number of different hormones, or possibly one hormone with a number of different functions. One stimulates the uterus to contract, another raises the blood pressure in mammals, and a third has a regulatory action upon the urinary secretion. The latter inhibits the secretion of urine in human beings, but on intravenous injection into anaesthetised animals it frequently exerts a diuretic action. It is therefore sometimes known as the diuretic-antidiuretic hormone. It is still uncertain, however, whether the pressor and antidiuretic activities are to be ascribed to different chemical substances. Abel maintains that all effects are due to a single compound, the molecule of which can be split into different parts, which each exert one or more of the activities of the original substance (*J. Pharm. Exp. Ther.* 1930, **40**, 139).

Commercial extracts are made from the posterior lobes of cattle or sheep. The oxytocic principle has been detected also in cerebrospinal fluid.

Preparation

A simple method of extraction is as follows:—The weighed finely minced posterior lobes are added to 0.5% acetic acid, and the

mixture is boiled for 10 minutes in a hard glass flask; after standing for at least 24 hours in the cold, it is filtered, made up to a definite volume, and sterilised again by heating to 90°. Albuminous matter can be precipitated by means of uranium acetate or by addition of dialysed iron, when the activity per grm. of solid is increased, though there is always a risk of loss by adsorption on to the precipitate.

The extract obtained by the above method contains the oxytocic, pressor, and antidiuretic principles. It cannot be identified by any known chemical means, and, indeed, of all the four hormones in common use, insulin, thyroxine, adrenaline, and pituitary (posterior lobe) extract, the last is the one concerning which we have the least amount of chemical knowledge.

Dudley (*J. Pharm. Exp. Ther.*, 1919, **14**, 295) describes a method of purification of the aqueous extract by use of colloidal iron followed by extraction with butyl alcohol.

1. The dried powdered posterior lobe is heated with 100 parts by weight of distilled water on a boiling water bath for 10 minutes. Two to three drops of *N*/2 acetic acid are added for every 10 c.c. of water, and the heating is continued for another 10 minutes. The mixture is cooled and filtered from precipitated protein.

2. Colloidal iron solution is added drop by drop to the above extract, the resulting precipitate being allowed to settle after addition of each drop. The supernatant liquid remains clear until excess has been added, when it turns brown. (About 0.2 c.c. of a solution containing 5% Fe_2O_3 to 5 c.c. of the extract is sufficient.) The method recommended by Dudley is to take 5 c.c. of the solution and add the colloidal iron solution until one drop causes it to turn brown. The rest of the solution is then added followed by the amount of iron calculated to be just sufficient to allow the solution to remain colourless. The mixture is heated for two minutes on the water bath, cooled, and filtered. By this method the solid in solution is reduced from 0.22 to 0.18%. It contains both the oxytocic and the pressor substances.

3. The aqueous solution is extracted by butyl alcohol in a special apparatus (see original paper). The butyl alcohol extract is evaporated in vacuo, water added, and the mixture redistilled until no butyl alcohol remains, the process is repeated, and finally the residue is dissolved in water, filtered, made up to 20 c.c., and tested. The solution contains an excess of the oxytocic principle.

O. Kamm, T. B. Aldrich, I. W. Grote, L. W. Rowe, and E. P. Bugbee (*J. Amer. Chem. Soc.*, 1928, **50**, 573) claim to have succeeded in separating from the extract two highly active solids which they call α- and β-hypophamine respectively, the first being 150 times as potent as the International standard powder in oxytocic activity, whilst the second has 80 times its pressor power; the diuretic-antidiuretic action appears to be a property of the second.

Their procedure is as follows:—

1. 100 grm. of commercial acetone desiccated gland material are extracted with 10 litres of 0.25% acetic acid, and the mixture is heated gradually to a temperature of about 95° for half an hour. It is cooled, filtered, and re-extracted with one litre of acid water, and the filtrates are combined.

2. The filtrate is concentrated at a low temperature in a current of air to a volume of one litre.

3. 550 grm. of ammonium sulphate are added, and the precipitate, which weighs 20–40 grm., is filtered off and dried.

4. Extraction with successive portions of glacial acetic acid (99–100%), using a total volume of 500 c.c., results in a separation of 5–8 grm. of active material.

5. 1250 c.c. of ether, followed immediately by 2500 c.c. of petroleum spirit, are added to the acetic acid extract, whereby the active fraction is precipitated. It is filtered off, washed with ether, and dried. Five to ten grm. of solid are obtained, representing about 80–90% of the total activity of the original desiccated gland, whether measured for the oxytocic or the pressor potency. A partial separation is then made.

6. Five grm. are dissolved in 250 c.c. of 98% acetic acid at a temperature of 40°, and precipitated by addition of 2.5 volumes of ether previously warmed to 30°. The precipitate is allowed to *settle for 5 minutes only, filtered off*, washed with ether, and dried. It is redissolved in 250 c.c. of 98% acetic acid, and the precipitation is repeated . . . (Ppt. 1).

The filtrates are combined, re-filtered through hardened paper, and treated with 10 c.c. of water and 2 volumes (about 3 litres) of petroleum spirit. After standing for several hours the clear liquid is decanted, and the gummy precipitate is dissolved in 50 c.c. of water and filtered free from fat . . . (Ppt. 2).

The activities of the two precipitates (expressed in international units per mg.) are given in the following table:—

	Precipitate 1	Precipitate 2
Oxytocic	5– 9 U per mg.	100–240 U per mg.
Pressor	10–18 U per mg.	3– 10 U per mg.

Purification of the Pressor Principle (Ppt. 1).—Five grm. are dissolved in 100 c.c. of 98% acetic acid at room temperature, and the solution filtered; the filtrate is treated as shown in the following table:

Filtrate
40 c.c. of acetone added

- Ppt. *mainly protein*. Some active material. Washed with ether and dried
- *Filtrate*. 40 c.c. acetone
  - 2nd protein fraction. Slightly more active
  - Filtrate. 25 c.c. ether
    - Ppt. filtered and dried. More active in both pressor and oxytocic potency
    - Filtrate. 125 c.c. ether added
      - Ppt. filtered and dried. Very active by pressor test. Less active by oxytocic test
      - Filtrate. Excess ether
        - Ppt. Remainder of pressor activity.
        - Filtrate. Most of the oxytocic activity with some pressor. Petroleum spirit
          - Filtrate
          - Oxytocic fraction

The final precipitate of the oxytocic fraction may occur in the form of a gummy mass, but by dissolving it in absolute alcohol and precipitating with excess of ether, it is obtained as a white powder.

The fractionation may be repeated on any of the precipitates. When the two fractions are mixed their action is similar to that of an

ordinary pituitary extract, showing that it is unlikely that any change has been caused by their separation.

J. J. Abel, C. H. Rouiller, and E. M. K. Geiling (*J. Pharm. Exp. Ther.*, 1923, **22**, 289) describe the preparation of a highly active tartrate.

1. Posterior lobes are ground to a fine paste; 100 grm. of the paste are thoroughly mixed with 100 c.c. of 0.35% hydrochloric acid containing 4 grm. of mercuric chloride. A bottle having a well fitting stopper is then filled with the solution, together with 10 grm. of solid mercuric chloride, and the mixture is shaken and filtered; the solid is washed twice by grinding with 25 c.c. of saturated mercuric chloride solution.

2. The mixture is ground with 200 c.c. of water, neutralised to litmus by addition of sodium hydroxide, treated first with hydrogen sulphide and then with 40 c.c. of saturated sodium chloride solution and filtered (the authors state that the filtration is very slow). The filtrate is aerated to remove hydrogen sulphide and treated with phosphotungstic acid until precipitation is complete. It is then filtered by suction.

3. Sodium carbonate is added to the phosphotungstate until it is just alkaline to litmus, and the phosphotungstate is re-precipitated with hydrochloric acid. This precipitation is repeated twice and the phosphotungstate dissolved in dilute sodium carbonate solution, cooled on ice, and hot barium hydroxide solution is added, drop by drop. The barium phosphotungstate is filtered off, and dilute sulphuric acid is added to the filtrate. After filtering, sodium hydroxide solution is added to neutralise the acid, followed by a concentrated solution of tannic acid and powdered sodium chloride until the solution is saturated. The tannate is filtered off and the process is repeated on the filtrate. The combined precipitates are washed several times by grinding with saturated sodium chloride solution and filtering.

4. *Conversion into tartrate.* The moist precipitate is ground with a little 95% alcohol and 1 or 2 c.c. of saturated alcoholic tartaric acid. The resulting solution is removed by means of a pipette, and the grinding repeated until the residue is practically colourless. About 20 volumes of anhydrous ether are then added to the extract, and the whole treatment with tartaric acid, alcohol, and ether is repeated three times.

5. The tartrate is ground with water, and a saturated solution of picrolonic acid in alcohol is added. The clear supernatant fluid is shaken with ether to remove the picrolonic acid and evaporated at room temperature. The residue is dissolved in 93% alcohol containing tartaric acid and much ether added. The tartrate is washed with a mixture of alcohol and ether (1:20) and dried over phosphorus pentoxide.

The preparation of a uniform powder for use as a standard in pituitary assay is carried out as follows (M. I. Smith and W. T. McClosky, *U. S. Public Health Service Hygienic Laboratory Bulletin* No. 138, Washington, 1924).

About 40 fresh posterior lobes, obtained within 10 or 20 minutes of the death of the animal, are dropped into a flask containing about 150 c.c. of acetone (40–60 glands yield 1.5–2.0 grm. of powder). The glands are dehydrated by this means and all enzymic action arrested. They are cut up into small pieces, again treated with acetone, and dried in a vacuum desiccator over calcium chloride at a temperature not exceeding 50°. The dried glands are then ground to a fine powder that will pass a No. 40 sieve. The small residue which cannot be ground (about 5%) is discarded. After further drying in the vacuum desiccator, the powder is extracted with acetone in a Soxhlet extractor for about three hours, and finally dried *in vacuo* at a temperature of about 40° to constant weight. The standard powder thus obtained is stored in a vacuum desiccator over calcium chloride out of the light; it represents about 16% of the original weight of the fresh gland.

Extracts of the standard powder are made for test by vigorously shaking 10 mg. of the powder with 10 c.c. of water for about ten minutes. The mixture is quickly boiled and filtered. 1 c.c. of the clear filtrate contains the activity corresponding to 1 mg. of the powder.

Properties

The activity of pituitary extracts is destroyed by *N* sodium hydroxide solution at room temperature, but it is more stable to sodium carbonate; it is also destroyed by boiling for 30 minutes with 0.5% hydrochloric acid.

The most outstanding character of the active principles of the posterior lobe is the readiness with which they are adsorbed on to

almost any other precipitate. It is this property that has caused the greatest difficulty in their isolation. Thus Dudley (*J. Pharm. Exp. Ther.*, 1923, **21**, 103) by treatment of the aqueous solution of his alcohol purified material with saturated sodium picrate, obtained a highly active crystalline substance which he was able to identify as the double picrate of creatinine and potassium with pituitary activity adsorbed. Dudley found that the aqueous solution of his butyl alcohol extract (mainly oxytocic activity) gave the following tests:—Millon's, glyoxylic acid and bromine, Molisch, Pauly; the biuret reaction was doubtful. The solution gave a slight amorphous precipitate with platinum chloride solution. Heavy precipitates were obtained with gold chloride, mercuric chloride, phosphotungstic acid, and a sticky precipitate with picric acid. The mixture was obviously a highly complex one. When alcohol was added to the aqueous solution a granular crystalline non-hygroscopic precipitate was formed having very little activity. Increasing the strength of alcohol brought out more activity, until finally the fraction almost completely soluble in alcohol could be precipitated with dry ether in the form of a yellow, very hygroscopic powder of great activity.

The following properties have been described more especially with regard to the oxytocic principle:—

1. In its original protein association it is insoluble in ether, petroleum spirit and chloroform, and practically insoluble in absolute alcohol. It is soluble in dilute acid and alkali.

2. It is destroyed more rapidly by lipase-free trypsin than by trypsin containing lipase, showing the probability of a peptide linkage; it is not destroyed by pepsin or papain.

3. The activity is quickly destroyed by hydrochloric acid in relatively low concentration, but acetic acid solutions are stable over a much wider range.

4. It is destroyed by alkali in the cold.

5. The maximum stability is found at *p*H 3.0–3.4. Purification does not alter the stability of the oxytocic principle.

Gaddum (*Biochem. J.*, 1930, **24**, 939) showed that, on heating in the region of strong acidity, the values for the potencies of solutions fall on a straight line the slope of which indicates a proportionality between the rate of destruction and the hydrogen ion concentration. At a strongly basic *p*H a linear relationship is also obtained, the rate of destruction being proportional to $(C_{OH})^{0.73}$.

The following table taken from Gaddum's paper shows the extreme care with which the *p*H of pituitary solutions must be adjusted before heating.

TABLE IV

	Temperature	Time	Activity left
	°C	Hours	%
*p*H 6.55	99	1.33	17.6
	80	16.0	1.39
	60	42.0	11.6
	50	69.2	33.8
*p*H 11.1	99	0.1	8.0
	74.5	0.783	3.0
	50	10.68	3.3
	25	47.67	33.3

Even at *p*H 3.5 heating to 100° causes 1% destruction in five minutes. A solution at *p*H 4.0 kept at 0° loses about 5% of its activity in one year.

The following represents all that is known with any degree of certainty concerning the pressor substance:—

1. It is less soluble in butyl alcohol than the oxytocic substance.
2. It is more easily decomposed.
3. The precipitate obtained by treatment of a phenolic solution of the crude posterior lobe extract with ether, contains an excess of the pressor substance.
4. The pressor substance is a physiological antagonist to insulin whilst the oxytocic is not.
5. It is destroyed by trypsin, but not by pepsin or papain.

In the opinion of most workers the diuretic-antidiuretic principle accompanies the pressor activity. According to C. Hogben and C. Gordon (*J. Exp. Biol.*, 1930, **7**, 286) sodium hydroxide increases the melanophore principle (which dilates the melanophores in amphibia), while destroying the pressor. The authors do not suggest the possibility of an isomeric change to account for this, but give a purely physiological explanation.

The tartrate described by Abel is very soluble in 93–95% alcohol, water, and pyridine. It is fairly soluble in methyl alcohol, but quite insoluble in dry ether, chloroform, acetone, and benzene. Ten per cent sodium hydroxide liberates a gas alkaline to litmus, and with a

smell resembling that of a mixture of an alkylamine with ammonia. A few drops of concentrated hydrochloric acid added to a minute amount warmed gently over a flame give a pink to violet colour.

In oxytocic activity it is claimed that the tartrate is active in a dilution of 1 in 18,750 million; 0.01 mg. exerts pressor activity in cats, and 0.05 mg. shows the diuretic action in rabbits.

Estimation

Only physiological methods of assay are available, and it is necessary to carry out separate tests for each activity. The same International standard is used for each one; from 0.5 mg. of this preparation 1 Unit of specific activity can be obtained (E. Knaffl-Lenz, *Publications of the League of Nations*, III. Health, 1928, III. 10. C.H. 734, pp. 48 & 68). The standard should be kept in a weighing bottle *in vacuo* over phosphorous pentoxide. For the test a small quantity is accurately weighed into a test tube, and 0.25% acetic acid is added, a volume of 1 c.c. being used for each mg. of powder taken. The test tube is plugged with cotton wool, heated on the boiling water bath for 3–10 minutes, and the extract, which contains 2 Units of activity per c.c., is filtered through dry paper. This extract is used as standard in each of the assays to be described.

Assay of Oxytocic Potency.—The excised uterus of the virgin guineapig is used as test object; the technique is described by J. H. Burn and H. H. Dale (*Reports on Biological Standards*, I. *Pituitary Extracts*. Medical Research Council: *Special Report* No. **69**, London, 1922). Attention must be directed particularly to the following points. Guineapigs weighing 200–250 grm. are the most suitable. They should have been kept away from contact with males from the time of weaning. The vagina should be closed, indicating that the animal is not in oestrus. It is killed by a blow on the head, and one horn of the uterus, together with the ovary at its upper pole is carefully dissected out without stretching it, the lower end being cut away from the vagina. It is then set up in a bath of warm oxygenated Ringer-Locke solution, the lower end being attached to a fixed hook, the upper hook passing through the ovary and being attached to a frontal writing lever. The bath, which should contain about 100 c.c. of Ringer-Locke solution, is surrounded by a water bath maintained at a constant temperature of 37°. The preparation is left undisturbed for about 20 minutes

during which time it relaxes to a constant length. A suitable uterus should show small irregular contractions. If these are large it should be discarded and a fresh one obtained. A suitable formula *for the Ringer-Locke solution is as follows:*

Sodium chloride	9	grm.
Potassium chloride	0.42	grm.
Calcium chloride	0.24	grm.
Magnesium chloride	0.005	grm.
Glucose	1.0	grm.
Sodium bicarbonate	0.5	grm.
Water (distilled)	1 litre	

It is absolutely essential that the salts and water used should be free from traces of the heavy metals.

The apparatus is designed so that the Ringer-Locke solution can be repeatedly run off and replaced by fresh solution at the same temperature. The lever should be provided with a Bowden cable, such as is used for the shutter release of a camera, to hold it steady during the time the solution is being changed, and should be weighted with ½–1 grm. The magnification need not be more than twice.

In carrying out the test the standard solution should be diluted 100 times. The first dose may be 0.5 c.c. of this dilution, that is, 0.01 U, but less or more may be required according to the uterus. A dose must be selected which gives a contraction slightly less than the maximum. Alternate doses of the standard and the solution under test are then added to the bath at regular intervals, which may vary in different experiments from 10 to 20 minutes. As soon as the contraction has reached its maximum after each addition the Ringer-Locke solution is changed twice, to wash away the active principle still remaining in the bath. The object of the test is the determination of a dose of the sample which gives a contraction equal to that produced by the dose of standard. Since the contractions given by the same dose may not always be exactly the same, confirmation of equality is obtained by finding the doses of sample which produce contractions slightly less than and slightly greater than that given by the dose of standard. In each case it is essential for an accurate assay, that two pairs of doses be used for the comparison, since the size of a contraction is frequently influenced by that of the preceding response, especially when the doses given are not exactly equal. It is sometimes advisable to add the doses in

the order: standard, sample, sample, standard, rather than alternately, and this order is recommended in the B.P. 1932. When the comparison has been carried out on six pairs of doses in this manner, the result may be considered accurate to within ±15%. Frequently a greater accuracy is attained.

The uterus of the guineapig also contracts to histamine, which may be present as an impurity in extracts of the posterior lobe. It can be detected by treating them with normal sodium hydroxide for one hour at room temperature, neutralising to litmus, and retesting. The oxytocic activity of the pituitary extract is destroyed by this treatment, any remaining stimulant power being due to the presence of histamine. Not more than 5% of the oxytocic power of the extract tested should be due to the latter, or not more than 0.1 mg. histamine should be present per 10 U oxytocic activity.

The British Pharmacopoeia 1932 describes this method for the biological assay of pituitary (posterior lobe) extract, and the United States Pharmacopoeia (Xth Revision) requires the use of the same method. The standard of the U.S.P., which is prepared from the posterior lobes of the pituitary bodies of cattle by the method of M. I. Smith and W. T. McClosky (*U. S. Public Health Service Hygienic Laboratory Bulletin* No. 138, Washington, 1924) has since been accepted as the International standard.

Assay of Pressor Potency.—This may be carried out on the cat under amytal, the decapitated cat, or the anaesthetised atropinised dog. Either of the latter methods is recommended by the Health Organisation of the League of Nations (*loc. cit.*). We have found it convenient to use the decapitated cat. The method of preparation is described under the physiological assay of adrenaline (see p. 381).

The object of the assay is the determination of the dose of the preparation under test which produces an exactly similar rise in blood pressure to that given by the selected dose of standard. Doses can only be given at infrequent intervals. L. T. Hogben, W. Schlapp, and A. D. McDonald (*Quart. J. Exp. Physiol.*, 1924, **14**, 301) recommend hourly injections, but we have found it possible to give them as frequently as every half hour. When given too frequently the preparation has not recovered from the effects of the previous dose, so that successive injections of the same dose produce successively smaller rises in blood pressure. Injections may be commenced about an hour after the destruction of the brain, when

the blood pressure has reached a constant low level. A suitable dose for injection into a 3–4 kg. cat is 0.25 unit. The dose of standard should be one which gives about half the possible maximum rise of blood pressure, and is given alternately with doses of the sample under test. The error of the assay may be taken as ±20%, but in many cases is not more than 10%.

Assay of Antidiuretic Potency.—Assays have been carried out on dogs, human beings, mice, and rats. The Health Organisation of the League of Nations recommends the use of the unanaesthetised dog, the normal man, or the patient with diabetes insipidus (*loc. cit.*). The method involving the use of rats described by J. H. Burn (*Quart. J. Pharm.*, 1931, **4**, 517) appears the most suitable. It is based on a method originally described by O. S. Gibbs for use with mice (*J. Pharm. Exp. Ther.*, 1930, **40**, 129).

Sixteen rats, weighing 120–140 grm. each, divided into four groups of four, are employed for an assay. Males give better results than females. The test is divided into two parts. Food is withdrawn from all the animals from 5 p.m. on the day preceding the test, but water is left in their cages. Each group is placed in a metabolism cage resting on a large funnel, and given warm tap water by mouth with the dose of pituitary extract subcutaneously immediately afterwards. Five c.c. of water per 100 grm. of body weight are administered by means of a gum elastic catheter passed through the central hole in a small wooden gag held in the animal's mouth. Two groups are injected with the standard extract, and two with the sample under examination, a suitable dose being 0.006 U per 100 grm. of body weight. The amount of urine excreted by each group is noted every 15 minutes, and the time taken to maximum excretion is thus determined. Two days later the test is repeated after a preliminary fasting period as before, but the two groups which were given the standard are now injected with the sample, and the two which were given the sample are injected with the standard.

The average times to maximum excretion for all four groups on standard and on sample are then determined. The relationship between the doses giving these effects is read from a curve relating dose to effect. This is obtained in the same way as the mouse insulin curve by experimentation on a large number of animals, using doses of standard ranging from 0.002 to 0.016 U per 100 grms. of body

weight, and is kept for reference (see under Physiological Assay of Insulin by the Mouse Convulsion Method for the use of such a curve). The error of the test is not more than $\pm 20\%$.

Uses and Toxicology

The oxytocic principle is used to stimulate the uterus during the course of, and at the end of, labour. It is especially valuable in post-partum haemorrhage. In small doses, such as 2 Units, it may be used without risk at any stage of labour to stimulate the uterine contractions.

The pressor principle is used in certain conditions of shock, to raise the blood pressure, and in cardiac failure to strengthen the heart beat.

The antidiuretic principle is specific in the treatment of diabetes insipidus. Polyuria is controlled by two injections daily.

Posterior lobe extracts have also been used in paralytic ileus to stimulate the smooth muscle of the intestine, in insulin hypoglycaemia to raise the blood sugar, in asthma, and in serum shock.

Solutions are generally given by subcutaneous injection, but may be applied to the nasal mucosa, or the dry powder may be administered in snuff.

An over-dose in labour may produce rupture of the uterus; in general it causes pallor, nausea, dyspnoea, and collapse, with fall of blood pressure, but these manifestations are not common.

PHARMACOPOEIAL PREPARATIONS

In Great Britain the Therapeutic Substances Regulations 1931 (Therapeutic Substances Act 1925) schedule pituitary posterior lobe extract. The acidity of the solution should lie between *p*H 3 and *p*H 4. The physiological tests must be carried out in comparison with the International Standard, and the potency of the solution expressed in terms of the International Unit. The date of manufacture should be specified on the label (Statutory Rules and Orders 1931 No. 633: Therapeutic Substances).

Pituitary posterior lobe extract is official in the British Pharmacopoeia 1932 as extractum pituitarii liquidum. Synonymous terms are liquor pituitarii, solution of pituitary or pituitary extract.

It is prepared by extraction with hot acidified water from posterior lobes dissected from glands frozen immediately after removal from

the animal. The filtrate is assayed and adjusted to the requisite strength and acidity, and sterilised by filtration or by heat. The solution must contain only a minimum amount of histamine.

Extractum pituitarii liquidum is identified by physiological tests: (a) oxytocic on the mammalian uterus, (b) pressor on the blood pressure of the mammal under the influence of a chemical anaesthetic or with its brain destroyed, and (c) antidiuretic on the mammal given water by mouth and the extract subcutaneously.

Pituitarium U.S.P. X (dried and powdered posterior lobe) is a yellowish or grayish amorphous powder having a characteristic odour. It is partly soluble in water. No tests are described. W. T. McClosky and J. C. Munch (*J. Amer. Pharm. Assoc.*, 1929, **18**, 31) recommend for the next edition that the potency be 50% of that of the standard powder.

The liquor pituitarii U.S.P. X is assayed physiologically for its oxytocic potency against a powder prepared by the method of Smith and McClosky (*loc. cit.*). The dosage recommended is 1 c.c. (that is 10 Units).

THE HORMONES OF THE ANTERIOR LOBE

Three distinct effects are ascribed to extracts of the anterior lobe of the pituitary gland, but there is much confusion as to whether they are due to separate substances, whether they may be due to one or two substances easily altered in physiological properties by a small change in chemical condition (*e. g.* by alteration in *p*H of the solution) or whether the different effects may be ascribed simply to a difference in size of the dose given. Whatever may be the true explanation, there is no doubt that extracts can stimulate growth in adult animals, produce premature sexual maturity in young animals, or cause cessation of oestrus in adult females by stimulation of the development of lutein tissue. The hormones which affect the sex glands (gonadotropic hormones) can also be extracted from the blood and urine of pregnant women and from human placenta.

The work of L. F. Hewitt (*Biochem. J.*, 1929, **23**, 718) supports the view that there are three separate and distinct substances for he has effected a rough separation.

The growth-promoting hormone together with that responsible for the development of lutein tissue is obtained by alkaline extraction and can be separated by subsequent treatment.

1. *Alkaline extraction.* Fifty fresh lobes are treated with 40% alcohol for 10 minutes at room temperature, rinsed 3 times with 0.9% saline, and ground with washed sand, a little water and 100 c.c. of 0.1 *N* sodium hydroxide solution being added. The mixture is allowed to stand overnight at 0°, neutralised to phenol red with 0.1 *N* hydrochloric acid and centrifuged for one hour. All manipulations should be carried out under aseptic conditions, and to provide a sterile solution for injection, the opalescent pink fluid is mixed with a sterile concentrated solution of 1.5 grm. of sodium benzoate. This extract contains the substances which stimulate growth and inhibit oestrus. By acidification, followed by filtration, the former property is liable to disappear. It can be separated from the latter by extraction with lime as follows:—

Fifty fresh lobes are ground with 300 c.c. of saturated lime water and the mixture allowed to stand. It is neutralised to phenol red by means of carbon dioxide, centrifuged after standing, and filtered through a Seitz filter. The resulting solution contains only the growth-promoting substance.

2. *Acid extraction.* Fifty lobes treated with 20 c.c. of water and 100 c.c. of 0.1 *N* hydrochloric acid for 18 hours at 0° and then centrifuged give an opalescent extract. If this is shaken with kaolin, centrifuged, neutralised with 0.1 *N* sodium hydroxide, centrifuged again, and put through a Seitz filter, the resulting solution contains the hormone which causes premature sexual maturity in females.

If the glands are extracted with acetic acid and the mixture centrifuged, neutralised and filtered through a Seitz filter, the resulting solution causes growth but has no effect upon the sex glands and organs of female animals. By treatment with kaolin after centrifuging, followed by dialysis of the solution, or subjecting it to ultrafiltration through cellophan, a solution is obtained which produces sexual maturity.

According to T. J. Putnam, H. M. Teel, and E. B. Benedict (*Amer. J. Physiol.*, 1928, **84**, 157) all three principles are present in the filtered neutralised product of alkaline extraction.

H. L. Fevold, F. L. Hisaw and L. S. Leonard (*Amer. J. Physiol.*, 1931, **97**, 291) claim to have separated the two gonadotropic hormones from a pyridine extract of the glands.

The extract made by stirring 5 grm. of the dried anterior lobe with 200 c.c. of aqueous 50% pyridine, centrifuging, and evaporating in air at 35°, gives a solid which, when emulsified with water, causes sexual maturity in young females and cessation of oestrus in adults.

On repeated extraction with distilled water the hormone producing follicular maturation passes into the aqueous solution, the luteinising hormone remaining in the residue. The authors give methods for purifying both, for which the original paper must be consulted.

H. M. Evans and M. E. Simpson (*J. Amer. Med. Assoc.*, 1928, **91**, 1337) recognise the possibility of separation of two hormones: (1) by alkaline aqueous extraction, a substance which stimulates growth in adults, (2) by acid aqueous extraction, one which provokes maturity in young animals. The second they found present also in alkaline and acid extracts of human placenta and in the urine of pregnant women.

Z. Wallen Lawrence and H. B. van Dyke state that sheep and pig pituitary glands give a better yield than ox glands.

M. Hill and A. S. Parkes (*Proc. Roy. Soc.* B, 1930, **107**, 30) have published the following method of extraction of the gonadotropic hormones from urine. The urine is evaporated to small volume at a temperature below 45°, washed out of the flask with a small amount of water and centrifuged. The precipitate is re-extracted with water, and four volumes of absolute alcohol are added to the combined extracts. The precipitate that forms is dissolved in water and again precipitated by a concentration of 80% alcohol. Oestrin being soluble in 80% alcohol is largely removed by this treatment, but any remaining in the precipitate is removed by successive extraction with absolute alcohol and acetone. A toxic substance may be removed by washing with chloroform. The fraction soluble in 30 to 50% alcohol was found to be active in immature mice, rabbits, and ferrets.

In other methods the hormone is precipitated from urine or from a neutralised alkaline extract of anterior lobes by means of sodium sulphate (See A. Biedl (*Endocrin.*, 1928, **2**, 241); E. P. Bugbee, A. E. Simond and H. M. Grimes (*Endocrin.*, 1931, **15**, 40); F. Dickens (*Biochem. J.*, 1930, **24**, 1507); H. M. Teel (*Science*, 1929, **69**, 405); and H. B. Van Dyke and Z. Wallen Lawrence (*J. Pharm. Exp. Ther.*, 1930, **40**, 413; 1931, **43**, 93)).

With so much diversity of opinion it is difficult to disentangle he chemical properties of substances extracted from the anterior obe of the pituitary gland, or even to say with certainty whether here are two or three to account for the three different effects bserved. The most reasonable view appears to be that expressed y Evans and Simpson with regard to their own extracts, namely, hat separation is not complete and that one hormone accompanies nother in greater or less amount according to the method of xtraction.

The following properties have been attributed to the three different principles. As work on the subject progresses they will no doubt e added to and modified.

The hormone producing sexual maturity is soluble in water, dilute odium hydroxide solution and pyridine, in 70% alcohol but not in 0%. It is insoluble in chloroform and ether. There is some loubt as to whether it can be dialysed through parchment or colodion, Hewitt having obtained active dialysates, whilst Wallen awrence and van Dyke state that it is not dialysable. It can be dsorbed on to animal charcoal or silica gel but not on to kaolin rom acid solution. Preparations from pituitary glands appear to e more stable than the substance isolated from the urine of pregancy. In the presence of acid it is more stable than the growthromoting hormone.

The growth-promoting hormone is soluble in water and dilute lkali, is adsorbed by kaolin from acid solution, and is unstable to cid and probably to pepsin and trypsin.

The hormone that arrests oestrus is soluble in water after treatnent with dilute sodium hydroxide. It is soluble in pyridine and 0% alcohol, but insoluble in acetone, 80% alcohol, and ether. According to Teel all three principles are destroyed by 0.5 N sodium ydroxide in 12 hours. The gonadotropic hormones can be disinguished from oestrin by the greater solubility of the latter in lcohol.

Physiological Assay

GROWTH PROMOTING HORMONE

Only qualitative methods are at present available, as no standard as yet been defined. An active extract when injected daily by the

intraperitoneal route into young adult rats produces a definite acceleration of the rate of growth as compared with control animals kept under exactly the same conditions. On cessation of the injections loss of weight occurs (E. P. Bugbee, A. E. Simond and H. M. Grimes (*Endocrin.*, 1931, **15**, 40); H. M. Evans and M. E. Simpson (*J. Amer. Med. Assoc.*, 1928, **91**, 1337); L. F. Hewitt (*Biochem. J.*, 1929, **23**, 718); H. B. van Dyke and Z. W. Lawrence (*J. Pharm. Exp. Ther.*, 1930, **40**, 413)).

GONADOTROPIC HORMONES

Only qualitative or roughly quantitative tests are available, as no standard has yet been defined. Young immature male or female mice or rats are employed.

In young immature female animals the gonad-stimulating hormone produces precocious sexual maturity which can be detected by premature opening of the vagina, the appearance of the squamous (cornified) cells of oestrus in the vaginal smear and some enlargement of the ovaries, owing to great enlargement of the follicles. The luteinising hormone has no effect on young immature animals, but injected with, or soon after, the gonad-stimulating hormone, it produces very great enlargement of the ovaries, which are filled with corpora lutea many of which may be haemorrhagic: the vagina opens prematurely but the vaginal smear shows only leucocytes. For test the extract can be injected subcutaneously twice daily for two up to five days (H. L. Fevold, F. L. Hisaw and S. L. Leonard, *Amer. J. Physiol.*, 1931, **97**, 291).

S. Janssen and A. Loeser (*Arch. exp. Path. Pharm.*, 1930, **151**, 188) recommend for the assay of the crude extract the use of a characteristic curve relating dose to effect produced with a suspension of powdered acetone-dried anterior lobe prepared according to the method of M. I. Smith and W. T. McClosky (*U. S. Public Health Service Hygienic Laboratory Bulletin*, No. 138, 1924). Groups of ten young rats, weighing 40–50 grm. each, are given six intraperitoneal injections in the course of 48 hours, and killed 100 hours after the first injection. The percentage showing corpora lutea or haemorrhagic points in the ovaries on each dose is determined. A curve relating dose to percentage response is constructed and used in the assay of other samples in the same manner as the curve relating dose to percentage of convulsions in mice in the assay of

insulin (see p. 401). Further information will be found in papers by A. Biedl (*Endokrin.*, 1928, **2**, 241) and B. Zondek (*Klin. Woch.*, 1930, **9**, 245, 393, 1207).

In immature male mice the injections produce hypertrophy of the seminal vesicles, which is detected by comparing the weight of the organs with those of control animals. The injections have little or no effect on the weight of the testes (F. Dickens (*Biochem. J.*, 1930, **24**, 1507); E. J. Kraus (*Klin. Woch.*, 1930, **9**, 1493); E. Dingemanse and S. E. de Jongh (*Pflügers Arch.*, 1930, **226**, 547)).

THE HORMONES OF THE SEX GLANDS

The sex glands, in addition to providing the specific sex cells, the spermatozoa and ova, also secrete hormones which control the development of the secondary sex organs and characters.

THE OVARY

The hormones of the ovary are responsible for the growth of the uterus and vagina and mammary glands at puberty, for the phenomena associated with heat or oestrus in lower animals or menstruation in women, and for the preparation of the uterus for the reception of the fertilised ovum and the maintenance of pregnancy to full term, as well as the coincident development of the mammary glands.

Oestrin, which is secreted by the cells of the ovarian stroma, and especially by the ripe Graafian follicles, produces the phenomena of oestrus when injected into spayed animals.

The cells of the corpus luteum which is formed in the ruptured follicle after extrusion of the ovum secrete hormones which bring about progestational proliferation of the uterine mucosa, provide for the continuance of pregnancy, and initate the full development of the mammary glands. Extracts of the corpus luteum also inhibit the oestrous cycle.

There is evidence that the cyclical nature of the reproductive process in female animals depends upon the activity of the anterior lobe of the pituitary gland.

OESTRIN

(Theelin, Theelol, Folliculin, Progynon, Menformon, "female sex hormone," "ovarian hormone")

Extracts which produce oestrus on injection into spayed animals have been obtained from the urine of pregnant women and human

placenta, from the ovaries and liquor folliculi of the cow and sow, and from other body fluids and tissues. The weight of the ovary depends greatly on the stage of the oestrous cycle at which it is removed; 10 grm. may be considered an average weight for the resting (dioestrous) gland in the cow and sow. Three different related substances have been isolated from the urine of pregnancy, two having oestrogenic properties, whilst no physiological action of the third has as yet been discovered. Crystalline preparations of the first two have been obtained by A. Butenandt (*Deut. Med. Woch.*, 1929, **55**, 2171), E. Dingemanse, S. E. de Jongh, S. Kober and E. Laqueur (*Deut. Med. Woch.*, 1930, **56**, 301), E. A. Doisy, C. D. Veler and S. A. Thayer (*Amer. J. Physiol.*, 1929, **90**, 329), G. F. Marrian (*Biochem. J.*, 1930, **24**, 435) and others.

The third compound isolated by Butenandt and by Marrian, and called by the latter "pregnandiol," is of no importance at the present time, except in so far as preparations of the two active substances are liable to be contaminated with it. Most of the investigators working upon the ovarian hormones have employed different names to designate the products which they have isolated, and some confusion has resulted. Quite recently, however, the differences between the two active compounds have been confirmed and satisfactorily explained by Butenandt and by Marrian, and the latter has suggested for them the names Trihydroxyoestrin and Ketohydroxyoestrin. For the sake of simplicity these names will be used here. Their relationship to the other substances described in the literature is made clear by the table.

TABLE V

	Trihydroxyoestrin			Ketohydroxyoestrin		Pregnandiol	
	Marrian (Oestrin)	Butenandt	Doisy and Thayer (Theelol)	Doisy and Thayer (Theelin)	Butenandt (Progynon)	Marrian	Butenandt
C %	74.76	74.72	75.12	76.69	79.68	78.08	78.64
H %	8.39	8.37	8.38	8.49	8.21	11.18	11.33
OH	3	3	3	1	1	2	2
C = O	0	0	0	1	1	0	0
M. Pt.	279.5–280.5°	276°	274°	249°	250–251° (corr)	233–234.5°	234–235°
Formula	$C_{18}H_{24}O_3$	$C_{18}H_{24}O_3$	$C_{18}H_{24}O_3$	$C_{18}H_{22}O_2$	$C_{18}H_{22}O_2$	$C_{19}H_{32}O_2$ or $C_{20}H_{34}O_2$	$C_{21}H_{36}O_2$

Trihydroxyoestrin can be converted into ketohydroxyoestrin by elimination of water, but it has been shown that they occur together

as separate substances in the urine, for both can be extracted from the same sample. The exact connection between these two and the saturated alcohol pregnandiol has not yet been discovered.

Constitution of Trihydroxyoestrin and Ketohydroxyoestrin

Trihydroxyoestrin contains three hydroxyl groups, one of which is acidic. Since it gives colour reactions characteristic of phenols and is soluble in sodium hydroxide but insoluble in sodium carbonate, the acidity must be due to a phenolic hydroxyl group. As the monomethyl ether is insoluble in sodium hydroxide, only one of the three hydroxyl groups is phenolic.

Ketohydroxyoestrin is formed from trihydroxyoestrin by elimination of water. Like trihydroxyoestrin, it is a weakly acidic substance, and owes its acidic nature to the presence of a phenolic hydroxyl group rather than to enolisation of the carbonyl group, as was at first suggested by Butenandt. This was shown conclusively by Marrian who on subliming in high vacuum the monomethyl ether of trihydroxyoestrin obtained a product identical with the monomethyl ether of ketohydroxyoestrin. Water must therefore have been eliminated between the two alcoholic hydroxyl groups. Furthermore, ketohydroxyoestrin methyl ether and the dehydrated product of trihydroxyoestrin methyl ether give identical oximes.

TRIHYDROXYOESTRIN

Preparation

In the latest method of preparation published by Marrian (*Biochem. J.*, 1932, **26**, 25) he incorporates into his process some of the operations described by Butenandt.

Urine is acidified with sulphuric acid and extracted 4 to 6 times with ether, the ethereal extract is evaporated, and the residue is treated with 5% aqueous potassium hydroxide for 2 hours. The alkaline mixture is saturated with carbon dioxide and extracted with ether. The residue, after evaporation of the ethereal solution, is boiled with dilute hydrochloric acid and, after dilution with water, extracted with ether, and the extract is washed with sodium carbonate solution and shaken with aqueous potassium hydroxide. The alkaline solution is saturated with carbon dioxide and extracted with ether, and the residue is again extracted from alkaline solution.

The final extract is dissolved in 60% ethyl alcohol and extracted several times with benzene, which removes the fraction containing ketohydroxyoestrin. The benzene is washed with 50% ethyl alcohol, and the washings are added to the alcoholic fraction, which is partly evaporated and extracted with ether, and the ethereal extract is evaporated to dryness. The residue is dissolved in ethyl acetate, and the solution is kept at 0° for 24–48 hours. The solid which separates is treated with charcoal in boiling alcohol, the solution is evaporated after filtration, and the residue is further purified by solution in *N* sodium hydroxide solution, followed by filtration and precipitation with carbon dioxide. The final product is recrystallised from aqueous ethyl alcohol.

Doisy and Thayer (*J. Biol. Chem.*, 1931, **91**, 641) have separated trihydroxyoestrin from ketohydroxyoestrin by taking advantage of the greater solubility of the latter in ethyl ether and in 95% alcohol.

Properties

Trihydroxyoestrin is a colourless crystalline substance occurring in two crystalline forms, plates or leaflets, and needles. It has a melting point of 279.5–280.5° (with slight decomposition) and is dextro-rotatory. Marrian gives the following values for the rotation

$[\alpha]_{5461}$ in pyridine $+41.9°$
$[\alpha]_{5461}$ in ethyl alcohol $+76°$

It has a physiological activity of 7,600 mouse units per milligram. The solubility in most organic fat solvents is low, but it is rather more soluble in methyl and ethyl alcohols, chloroform and acetone, than in ether. It is soluble in pyridine, and, being weakly acidic in character, will dissolve completely, though slowly, in cold 5% potassium hydroxide solution, but is displaced from its alkali salts by carbon dioxide.

It is non-volatile and only a very small amount will sublime at a temperature of 165° and a pressure of 0.001 mm. mercury. Trihydroxyoestrin gives Millon's reaction, and also turns yellow with strong nitric acid, giving a yellow solution.

KETOHYDROXYOESTRIN

Preparation

1. In the most recent method of preparation described by Veler, Thayer and Doisy (*J. Biol. Chem.*, 1930, **87**, 357), urine of pregnancy

is extracted with butyl alcohol in a specially constructed extraction apparatus (for this the original paper must be consulted; the apparatus is capable of extracting 25–30 gallons per 24 hours without any skilled attention). 1,000 c.c. are distilled, and the residue is extracted by heating and shaking with 400 c.c. of benzene, followed by two 200 c.c. portions. The combined extracts are chilled, the small precipitate is removed, the benzene solution is distilled, and the tarry residue is dissolved in 50 c.c. of butyl alcohol and 1,000 c.c. of petroleum spirit. This is shaken successively with 400 c.c. of 0.5 N sodium hydroxide solution, then with 400 c.c., 200 c.c., and 200 c.c. of 0.25 N sodium hydroxide solution, and the fractions are mixed. The alkaline solution is partly neutralised and extracted with ethyl ether, the ether is removed, and the residue is steam-distilled. The condensed steam is removed from the product by distillation, and the tarry residue is extracted 4 or 5 times with 25 c.c. of hot 0.25 N sodium hydroxide solution. The combined extracts are filtered through a Jena glass filter (porosity < 7) and extracted 6 times with a fourth of its volume of ether; the ethereal extracts are washed once with 20 c.c. of 0.1 N sodium bicarbonate solution, once with 20 c.c. of 0.1 N hydrochloric acid, and once with water. The ether is distilled, and the residue is dried in *vacuo* and extracted with 20 c.c. cold (25°) 0.25 N sodium hydroxide solution. The alkaline extraction is repeated 4 or 5 times, the residue being treated between every extraction with 2 c.c. of ethyl alcohol and distilled so as to expose a new surface for extraction. The aqueous extract is next extracted 4 or 5 times with a fourth of its volume of ether, and the combined ethereal extracts are washed as before with sodium bicarbonate solution, hydrochloric acid, and water. The ether is distilled, a few c.c. of ethyl alcohol are added, and the liquid is distilled to dryness. The residue (a light yellow oil containing crystals) is re-crystallised from solution in aqueous alcohol, butyl alcohol, and petroleum spirit. Re-crystallisation is repeated until the product melts at 243° (heated slowly).

2. Butenandt's method for obtaining crystals from the crude oil extracted from urine of pregnancy is shown in the chart taken from his paper (*Gött. Abh. Math. Phys. Kl.*, III Folge, Heft 2, p. 48, 1931).

CRUDE OIL FROM URINE OF PREGNANCY
(30 Mouse Units (M.U.) per mg.)

Treated with 50% methyl alcohol and petroleum spirit

Alcohol fraction
Add water and extract with ether (100–200 M.U. per mg.)

Petroleum spirit fraction

Treat with 70% alcohol and benzene

Benzene fraction (300–500 M.U. per mg.). Treat with dilute HCl and extract with ether

Alcohol fraction

Ethereal solution
Treat with sodium carbonate

Carbonate soluble portion

Ethereal residue
Treat with sodium hydroxide

Alkaline solution. Acidify and extract with ether (1,500–2,000 M.U. per mg.) Fractionally distil in high vacuum.

Alkali insoluble inactive fraction. Pregnandiol m.p. 235°

Crude crystalline material (7–8,000 M.U. per mg.). → Pure crystalline hormone m.p. 250–251° (8,000–10,000 M.U. per mg.)

3. *Preparation from Trihydroxyoestrin.*—Marrian describes the following procedure: 0.66 grm., mixed intimately with 16 grm. of powdered fused potassium acid sulphate, was heated in a small retort for 5 hours at 110°, 3 hours at 140°, and 6 hours at 150°, at a pressure of 0.02 mm. mercury. The potassium acid sulphate was separated from the mixture by solution in $N/100$ sodium hydroxide and extraction with ether. 0.22 grm. of the crude product was obtained, representing about 78% yield of activity.

Properties

Ketohydroxyoestrin is a colourless crystalline substance occurring like trihydroxyoestrin, in two crystalline forms. According to

Doisy, Veler, and Thayer, the crystalline form depends upon the solvent and method of obtaining the crystals. Thus, extraction with ether from alkaline solution gives leafy crystals, but flat platelets of rhombohedral shape are obtained by precipitation from ethyl alcohol with distilled water. Butenandt describes also branching crystals, 1–2 cm. long, with a lustre like that of mother of pearl, obtained by slow crystallisation from dilute alcohol.

The melting points quoted are:—

Marrian	242–246°
Doisy, Veler and Thayer	249°
Butenandt	250–251°
Dingemanse, de Jongh, Kober and Laqueur	240°

Marrian gives the rotation $[\alpha]_{5461}$ + 188° in ethyl alcohol
Butenandt gives $[\alpha]_D^{18°}$ + 156° in chloroform

The ultra-violet absorption spectrum shows a characteristic absorption with a maximum at 283–285 Å.

Like trihydroxyoestrin, it is a weakly acidic substance and owes its solubility in sodium hydroxide solution to a phenolic hydroxyl group. It is readily soluble in alcohol, acetone, chloroform and benzene, less soluble in ether and ethyl acetate, and sparingly soluble in petroleum spirit. From solution in alcohol and acetone it can be precipitated with water, from solution in ethyl acetate, chloroform, or benzene, by petroleum spirit. It is very insoluble in water, not more than 1.5 mg. dissolving in 100 c.c.

It can be distilled at 130–150° at 0.01–0.03 mm. mercury. Ketohydroxyoestrin has a physiological activity of 17,800 mouse units per mg. Ketohydroxyoestrin and trihydroxyoestrin can be distinguished from each other by their different melting points, optical rotations, and physiological activities, and by the difference in rotation of their monomethyl ethers and the melting points of some of their salts.

DERIVATIVES

Trihydroxyoestrin acetate, $C_{18}H_{21}(O.CO.CH_3)_3$, is a white crystalline substance of m.p. 120–122° (Marrian), 126° (Doisy and Thayer), having a physiological activity about half as great as that of the parent substance (3,700 mouse units per mg.).

It is prepared from trihydroxyoestrin as follows:—About 0.02 grm. of trihydroxyoestrin is heated with 2 c.c. of acetic anhydride to 110°

for two hours. Upon adding excess of water and scratching the surface with a glass rod a white solid separates. The precipitate of the impure acetate can be purified by solution in hot alcohol and evaporation in a vacuum desiccator over solid potassium hydroxide. The clear gum obtained is subjected twice again to the same treatment, dissolved in 1 c.c. of hot methyl alcohol, and hot water is added, drop by drop, until a faint cloudiness appears. Upon standing at 0° rosettes of white needles are formed, which can be recrystallised from alcohol by addition of water. According to Doisy and Thayer it can be distilled unchanged at 210° and 0.026 mm. mercury. By hydrolysis with alcoholic sodium ethoxide the parent substance can be recovered.

Trihydroxyoestrin Monomethyl Ether, $C_{18}H_{23}O_2.OCH_3$, has been prepared in crystalline form by Marrian and by Butenandt. It has m. p. 162.5–164°, according to Marrian, who prepared it by treatment with dimethylsulphate in alkaline solution, and 169° according to Butenandt, who prepared it by action of diazomethane. The rotations given are:—

$[\alpha]_{5461}$ + 29.9° in pyridine (Marrian)
$[\alpha]_{5461}$ + 65.3° in ethyl alcohol (Marrian)
$[\alpha]_D$ + 34.7° in pyridine (Butenandt)

Doisy and Thayer state that it can be distilled at 220–230° at 0.02 mm. mercury.

Ketohydroxyoestrin Monomethyl Ether, $C_{18}H_{21}O.OCH_3$, is a crystalline substance described by Butenandt and by Marrian; it has m. p. 164–168°, and rotation $[\alpha]_{5461}$ + 171° (in chloroform). The preparation is described by Marrian as follows: 0.030 grm. of ketohydroxyoestrin was dissolved by warming in 20 c.c. of $N/2$ sodium hydroxide solution and shaken with 0.5 c.c. of dimethyl sulphate for 2 hours; the precipitate was filtered off, suspended in 20 c.c. of $N/2$ sodium hydroxide solution and warmed for one hour. The insoluble residue was washed, dried, and recrystallised from methyl alcohol after treatment with charcoal in ethyl alcohol.

Ketohydroxyoestrin Oxime. The oxime of ketohydroxyoestrin according to Marrian has m. p. 182–187°, but Butenandt gives a m. p. of 233°.

Dihydrodesoxyoestrin, $C_{18}H_{25}OH$, prepared by Butenandt by reduction of ketohydroxyoestrin, has m. p. 129°.

Other derivatives of these two substances mentioned in the literature are:—

Ketohydroxyoestrin semicarbazone	m. p. 258°
Ketohydroxyoestrin acetate	m. p. 126°
Ketohydroxyoestrin benzoate	m. p. 214°

PREGNANDIOL

$C_{21}H_{36}O_2$ (Butenandt)

or

$C_{19}H_{30}(OH)_2$ or $C_{20}H_{32}(OH)_2$ (Marrian)

is a saturated alcohol which always accompanies the oestrogenic hormones in urine of pregnancy. It occurs in the non-saponifiable fraction of the ether-soluble material. It is a white crystalline substance of m. p. 233–235°. Pregnandiol has a low solubility in all common organic solvents. It is somewhat more soluble in cold benzene, toluene, ethyl alcohol, methyl alcohol, and chloroform, than in ether, petroleum spirit, or cold acetone, but in all cases the solubility is less than 1%. It is completely insoluble in hot and cold water and in aqueous alkali.

Pregnandiol Acetate, $C_{19}H_{30}(O.CO.CH_3)_2$ or $C_{20}H_{32}(O.CO.CH_3)_2$ (Marrian), has m. p. 177.5–178.5°. It is much more soluble than the parent substance and dissolves readily in cold ethyl alcohol, chloroform, and acetone; it is only slightly soluble in cold methyl alcohol, but dissolves on heating.

Physiological Assay

No standard has yet been officially adopted for oestrin, but for accurate quantitative work a pure specimen of the compound under examination, trihydroxyoestrin or ketohydroxyoestrin, should be used as the standard. Units have so far been defined in terms of an animal reaction, usually the production of oestrus in spayed mice or rats. The value depends on the medium in which the oestrin is given, oil or water, on the number of injections, and the interval between them. It is also influenced by the particular reaction taken as the end-point, and the number of animals out of those injected which are required to show this degree of response. It is customary to specify the species of animal employed. According to the conditions of the test the Rat Unit may be the same as the Mouse Unit or up to 8 times larger. Results therefore obtained in the same laboratories may be comparable, but it is difficult to

correlate them with figures given by other workers, unless the same technique is rigidly employed. The need for a stable standard must therefore be emphasised.

Daily vaginal smears are taken for two weeks from young adult animals from a uniform stock maintained on a constant diet at a constant environmental temperature, and all animals that do not show normal oestrous cycles are discarded. The operation of oöphorectomy is then carried out under ether anaesthesia. The animal is placed on its belly, a small incision made in the loin to one side of the mid line, between the lowest rib and the pelvis, and carried through subcutaneous tissue and muscle until the abdominal cavity is opened. The ovary is found in a mass of fat which can be lifted out of the wound, and is excised together with the tube (oviduct) and a portion of the uterus and fat. In the rat it is necessary to apply a ligature before cutting the uterus, but in the mouse this is not essential. The wound is closed with one or two ligatures or a Michel clip. The operation is then repeated on the opposite side. Its completeness is determined by making vaginal smears for a fortnight, commencing about a week after the operation. No oestrous cycles should be observed.

Vaginal smears are made by passing a sterilised small platinum loop into the vagina and sweeping it round the walls. The loop is then rubbed in a drop of water on a slide, and the smear is immediately examined under a low power of the microscope. Staining is not necessary. In a spayed animal a smear consists chiefly of leucocytes with a few nucleated epithelial cells. The complete response to an injection of an active preparation is the disappearance of the leucocytes and nucleated epithelial cells, the smear consisting exclusively of large non-nucleated squamous (cornified) cells. As this stage may be of very short duration and so may not be observed, the disappearance of leucocytes with the appearance of nucleated or nucleated and squamous cells on one day, followed by a mixture of leucocytes and a large number of squamous cells on the next day, may be taken also as a positive response.

The result of the assay is greatly influenced by the number of injections given. With a solution in oil, a single subcutaneous injection suffices, but with an aqueous solution, the dose necessary to produce a complete response is very much larger than when the injections are made over the course of one or two days. Whatever

technique is adopted, it must be adhered to throughout. For aqueous solutions six injections over a course of 48 hours, *i.e.* two on the first, three on the second, and the last on the morning of the third day, may be given. The volume for injection in the case of the mouse should be 0.1 c.c. and for the rat 0.5 c.c. Vaginal smears are taken on the morning of the third, fourth, and fifth days, as well as on the evenings of the third and fourth days.

For roughly quantitative tests it is sufficient to employ about ten animals and to observe the number which give a positive response, the dosage being such that 50–75% respond. For all accurate assays a curve relating dose to percentage response should be obtained with the standard preparation, as described for the assay of insulin with mice (see p. 401). Not less than five points on this curve must be determined, at least 100 animals being used for each point. Within the range of about 30–75% response, 40 animals (20 on standard and 20 on sample) will give a result within 10% of the true value when the potency is determined with the use of the characteristic curve, as described under insulin.

Animals can be used weekly or fortnightly. Within limits age and weight make no difference to the response.

The following papers should be consulted for further details of the test:—

H. Allan, F. Dickens, and E. C. Dodds, *J. Physiol.*, 1930, **68**, 348.

A. Butenandt. Untersuchungen über das Weibliche Sexualhormone, 1931; *Gött. Abh. Math. Phys. Kl.* III. Folge, Heft 2.

K. H. Coward and J. H. Burn. *J. Physiol.*, 1927, **63**, 270.

J. M. Curtis and E. A. Doisy. *J. Biol. Chem.*, 1931, **91**, 647.

F. E. d'Amour and R. G. Gustavson. *J. Pharm. Exp. Ther.*, 1930, **40**, 473, 485.

L. C. Kahnt and E. A. Doisy. *Endocrin.*, 1928, **12**, 760.

W. P. Kennedy. *Quart. J. Exp. Physiol.*, 1930, **20**, 71.

E. Laqueur and S. E. de Jongh. *J. Pharm. Exp. Ther.*, 1929, **36**, 1.

G. F. Marrian and A. S. Parkes. *J. Physiol.*, 1929, **67**, 389.

Use

Oestrin has been found of value in certain cases of secondary amenorrhoea and dysmenorrhoea. It is given by the hypodermic route since oral administration necessitates the use of much larger doses.

THE HORMONES OF THE CORPUS LUTEUM

(Progestin, Relaxin, Lutéocrinine, Lutein)

Active extracts are usually obtained from the corpora lutea of the sow or cow. Relaxin has been found in the blood or placenta of some species during pregnancy.

Various methods of preparation are described by different workers, and the chemical properties of the active substances appear to differ, so that it is probable that the various physiological functions of extracts are due to different hormones. None have yet been obtained in a pure state.

Properties

Progestin, or the hormone which causes progestational proliferation of the uterine mucosa is, according to W. M. Allen, soluble in 95% ethyl alcohol, methyl alcohol, acetone, ether, benzene and chloroform (*Amer. J. Physiol.*, 1929, **92,** 174). The method of extraction is based on these properties. The purified hormone is also soluble in water and dilute acid. It is destroyed by strong alkali and prolonged heating, but not by mild oxidation. Of the solvents available, it is most stable in 95% alcohol. Fevold and his co-workers (*vide infra*) state that their preparation is only slightly soluble in ether.

Relaxin, or the hormone which produces relaxation of the pelvic ligaments of the guineapig, resembles insulin in its properties, and is extracted from the ovaries with acid alcohol (H. L. Fevold, F. L. Hisaw, & R. K. Meyer, *J. Amer. Chem. Soc.*, 1930, **52,** 3340; *Proc. Soc. Exp. Biol. & Med.*, 1929, **27,** 606). It is slightly soluble in water and 95% alcohol, but insoluble in absolute ethyl alcohol and other organic solvents. It can be separated from progestin by its insolubility in 99% ethyl alcohol. It is soluble in dilute acid and alkali, and is stable at *p*H 3.2 or in acid alcohol, but is destroyed at *p*H 9 and by mild oxidation or heat, and by pepsin and trypsin.

Lutéocrinine, which inhibits the oestrous cycle, is obtained by P. Gley by extraction with 1% tartaric acid, and is soluble in water (*J. Physiol. et Path. gén.*, 1929, **27,** 528). The paper by D. I. Macht, A. E. Stickels and D. L. Seckinger may also be referred to (*Amer. J. Physiol.*, 1929, **88,** 65).

Physiological Assay

Only roughly quantitative tests are available for the estimation of these hormones. They are based on the power to stimulate growth of the uterine mucosa (C. Clauberg, *Klin. Woch.*, 1930, **9**, 2004), or abolish the sensitivity of the uterine muscle to pituitary (posterior lobe) extract (H. Knaus, *Arch. exp. Path. Pharm.*, 1930, **151**, 371), or to relax the pelvic ligaments (H. L. Fevold, F. L. Hisaw and R. K. Meyer, *J. Amer. Chem. Soc.*, 1930, **52**, 3340), or to inhibit oestrus (A. S. Parkes and C. W. Bellerby, *J. Physiol.*, 1927, **64**, 233; G. N. Papanicolaou, *J. Amer. Med. Assoc.*, 1926, **86**, 1422; J. S. Patel, *Quart. J. Exp. Physiol.*, 1930, **20**, 245).

Rats, rabbits, or guineapigs are used. The method described by Knaus for the estimation of the potency of alcoholic extracts (progestin) is as follows:—The extract under examination is injected subcutaneously into a rabbit or a series of rabbits in different doses, four injections being given in the course of 36 hours. Twelve hours later the uterus is excised and set up in a bath of warm oxygenated Ringer-Locke solution, and its response to the addition to the bath of 0.5 Unit of pituitary (posterior lobe) extract is determined. An active extract, when given in sufficient doses, will abolish the contraction of the uterus normally produced by pituitary (posterior lobe) extract.

Use

Extracts of corpus luteum have been of value in arresting the excessive bleeding in menorrhagia.

THE TESTIS

The internal secretion of the testis is responsible for the growth of the penis, seminal vesicles and other secondary sex organs in the male which occurs at puberty, and is essential for the maintenance of these structures in their normal adult condition. The hormone is secreted by the interstitial cells of Leydig, which lie between the seminiferous tubules.

Extracts containing the active principle have been obtained from the testicular glands of the bull, pig, and ram, from the epididymis and blood of the bull, and also from human urine. The testis of the

bull weighs 250–300 grm. after removal of the outer fibrous coat (*tunica albuginea*).

Preparation

T. F. Gallagher and F. C. Koch (*J. Biol. Chem.*, 1929, **84**, 495; *J. Pharm. Exp. Ther.*, 1930, **40**, 327) extract minced testes with 95% alcohol; the extract is concentrated and treated with benzene. The benzene extract is separated, the benzene is removed at low temperature and pressure, and the residue is extracted with cold acetone. The acetone-soluble fraction is dissolved in 70% alcohol, and impurities are removed by extraction with hexane. The alcohol is removed, the residue is taken up in ether, and the ethereal solution is washed in turn with alkali, acid, and water.

The active principle is extracted from male urine after acidification by treatment with ether or benzene. The ethereal solution is washed with alkali, which removes inert matter, and then evaporated; the residue is taken up in hexane and the solution is again washed with alkali.

E. C. Dodds, A. Greenwood, H. Allan, and E. J. Gallimore (*Biochem. J.*, 1930, **24**, 1031) described a method involving the use of hot barium hydroxide, followed by extraction with butyl alcohol.

Properties

The testicular hormone is soluble in organic solvents, aqueous alcohol and liquid ammonia, but only slightly soluble in water. It is not soluble in alkali. It resists mild reduction in acid or alkali and treatment with dilute acid. It is destroyed by prolonged action of sodium hydroxide.

A. Butenandt (*Z. angew. Chem.*, 1931, **44**, 905) has obtained from human male urine a crystalline compound of high activity, which has the empirical formula $C_{16}H_{26}O_2$ and melts at 178°. An oxime of melting point 215° has been obtained by treatment with hydroxylamine. From this the active principle can be obtained by acid hydrolysis and fractional sublimation in a high vacuum at 80–85°. The oxime has a prolonged physiological action probably due to hydrolysis in vivo. The active compound is a neutral substance containing at least one hydroxyl group, removal of which results in loss of potency.

Gallagher and Koch (*loc. cit.*) have obtained acetyl, benzoyl and phenyl-urethane derivatives of lower potency than the original compound.

Physiological Assay

A number of qualitative tests are available for the detection of the testicular hormone, one of which can also be considered as fairly quantitative. An active extract prevents the atrophy and accompanying histological changes of the accessory sex organs in young castrated rats, mice, or guineapigs; it also prevents loss of motility of the spermatozoa (C. R. Moore and T. F. Gallagher, *Amer. J. Physiol.*, 1929, **89**, 388; *J. Pharm. Exp. Ther.*, 1930, **40**, 341). In capons it stimulates the growth of the comb, a reaction which is capable of being employed in a quantitative manner (J. S. S. Blyth, E. C. Dodds and E. J. Gallimore, *J. Physiol.*, 1931, **73**, 136; T. F. Gallagher and F. C. Koch, *J. Pharm. Exp. Ther.*, 1930, **40**, 327). Birds may be castrated from soon after hatching up to two months of age. Five daily intramuscular injections of the extract under examination are given, and the increase in size of the comb is estimated from its increase in length and height, or from the increase in surface area as measured by the planimeter. A curve can be constructed relating increase in size of the comb to dose given, and used for reference in later tests. There is at present no standard, and samples should be assayed against an arbitrarily selected standard for which a curve must be constructed. Provided the growth obtained is submaximal, and exceeds 15% of the original size (to exclude spontaneous variations), the error of the assay is about ±30%, when five birds are injected with the sample and five with the standard.

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THE IDENTIFICATION OF UNKNOWN WOODS AND CHARCOALS

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INTRODUCTORY

During the past century, there has slowly emerged from the laboratory of the botanist, from the museum of the collector, from the workshops of joiner and timber merchant, and from the field-practice of the silviculturalist yet another special science—the science of Wood Technology. In this connection, because of its direct economic, forensic and antiquarian applications, a more limited branch of learning, which may be termed the science of Wood Anatomy, has already earned for itself a discrete importance.

The present article is concerned primarily with problems of wood identification. Wood identification, however, is often so intimately bound up with other lines of forest products research that it will be as well first to enumerate the latter.

Forest Products Research.—Within this category may be included various affiliated sciences, namely the Mechanics, Seasoning, Preservation, Machining and Hand-working, Mycology, Entomology, Physics, Chemistry and Anatomy of wood; the last representing both the *alma mater* and hand-maiden of all the rest, since the properties of a wood depend upon its structure, whilst its genealogy and individual growth-history also are therein recorded.

Then, in addition to such special subjects of investigation, there are the immensely important problems of practical silviculture, timber utilisation and taxonomical classification requiring expert attention.

Analysis and Identification of Woods.—Considered chemically and physically, as well as anatomically, wood is an exceedingly

complex substance, and its detailed chemical analysis, therefore, presents a problem of the greatest difficulty. An approximate estimation of the relative proportions of sugars, starches, lignins (or lignones), celluloses, ligno-celluloses, pecto-celluloses, hemicelluloses, tannins, etc., however, may sometimes be found to be of specific and economic, as well as academic, interest. Still more so, the presence of characteristic oils, resins, dyestuffs, alkaloids, etc., should be considered; while physical properties such as specific gravity, relative hardness, colour, smell and mechanical strength—all estimated according to agreed standard methods—may also be of occasional assistance to the anatomist in his attempts to identify an unknown wood specimen.

Finally, although a given tree species may generally be more securely designated in terms of leaves, flowers, fruit, bark and external form, than from its wood anatomy, there do sometimes arise instances of doubt in which the wood structuralist can aid the systematic botanist.

Hence, we see that Wood Identification does not stand by any means isolated from other academic and practical sciences. Apart from certain obvious properties, such as specific gravity, colour, hardness, smell and the possession of particular oils, dyes, alkaloids, or other specific substances, however, anatomical structure will usually be found to afford the most convenient basis for diagnosis.

For many years past sporadic requests have been received at botanical laboratories, museums and herbaria, from individuals, firms and public institutions, requesting the identification of specimens of wood or charcoal, and, more rarely, even fossilised wood. Occasionally, too, considerable collections of useful or decorative timber samples have been built up by solitary enthusiasts. But it was not until comparatively recently that the subject began to attract public or state attention.

Scientific forestry has been practised in Europe during several centuries, and was actually being taught in Germany and France at the beginning of the 18th. century. Not so the organised investigation of forest products, however, until the past few decades witnessed the foundation of special research stations (the old Cooper's Hill College in England and the Madison Forest Products Laboratory, U. S. A., for instance) in all the principal civilized countries of the world. One of the determining factors in this respect was a

threatened world shortage of softwood and other timbers, which promised to become more acute after the World War of 1914–18. Many such research stations possess considerable collections of authentic commercial wood species, systematically classified and conveniently arranged for immediate reference or comparison—a basic necessity for the serious wood anatomist. Again, many of these institutions are now open to public service: giving advice in connection with all technical and scientific timber problems, carrying out tests, analyses and identifications for their clients, either free or upon payment of a moderate fee.

In addition to those of more general scope, such as the Kew (Jodrell) Laboratory, the Imperial Institute and the Natural History Museum, London, we now have two more specialised national institutions in the British Isles, namely, the Imperial Forestry Institute, Oxford[1] and the Forest Products Research Laboratory, Princes Risborough, Bucks, at which timber science is studied and applied in all its chief branches. In addition, of course, identifications and other structural work can also be carried out by individual workers upon a somewhat more limited scale; one of the aims of this article being to show broadly the manner in which that can be done; more especially as specimens of wood or charcoal occasionally come into the hands of the public and commercial analyst, in connection with various legal and economic investigations. As the science of Wood Anatomy, however, is as yet comparatively young, and the special technique in process of continual modification and improvement, only certain widely accepted methods will be described.

A list of reliable text books on the subject is also appended, and the possible value of certain chemical and physical properties is briefly discussed.

The choice and preparation of material for identification will be treated first, and will be followed by a general account of anatomical identification procedure.

NORMAL WOODS

Choice of Material.—The choice of material for detailed examination or analysis will depend, first, upon the size, and second, upon

[1] In conjunction with the older University School of Forestry, one feature of the Institute being the training of probationary forest officers for British colonial governments. Research is also carried out, and there is a fine wood collection of several thousand separate wood species—many samples in duplicate—rapidly expanding annually.

the physical condition of the original sample. In speaking of fresh and normal woods, however, we may omit the latter consideration. Partly decayed or charred samples will be considered separately below.

On the other hand, samples are frequently of such a size or shape that no subsequent choice of material is possible; and some such fragments—*e. g.* of less than 1 c.c. in volume—may be found unsuitable for reliable specific identification; for the majority of non-tropical woods have their tissues segregated into successive zones, the omission of any one of which may render a diagnosis worthless. Certain tropical hardwoods, a few colder-climate species and some softwoods, however, possess a texture sufficiently fine and homogeneous to permit of identification from relatively minute specimens.

In order, therefore, to render an identification reasonably secure, it is usually desirable to obtain a good-sized sample, so that a considerable area may first be examined with the unaided eye, a low-power hand lens, or a large-field binocular microscope. Subsequently, a picked sample can be extracted from the larger block for microscopic examination or chemical treatment.

In general, it will be found convenient to cut the sample blocks for microscopic examination not less than 0.5 inch square (on the transverse section) by 0.75 inch long. This size gives sections sufficiently large for most purposes, a set of three of which (*viz.*, transverse, tangential-longitudinal and radial-longitudinal) may be mounted on a single micro-slide under a large cover-slip. Blocks should normally be cut square to the grain, as oblique sections are difficult to interpret.

In addition to the insufficient size of the original sample, its nature may also be unsuitable. For instance, knots and traumatisms in the wood may produce deformation of the neighbouring tissues, and other abnormal structures may be encountered. Such defective material should, if possible, be discarded. More confusing still, as being less easily recognised and guarded against, is the atypical wood frequently met with in small twigs and branches. Wood from the bole, or main stem of a tree, therefore, is always preferable. Small branches, as also late-formed and senile wood from the boles of old trees, often possesses very narrow growth-rings, in which the specific structure is less easily identified than in better grown specimens. In the former type of wood, crowding together of the

"pores" (*i. e.*, conducting vessels) may produce an altogether atypical appearance. Again, in extra narrow-ringed and badly grown samples of a wood such as common European oak, the typical zones of thick-walled strengthening fibres may be almost or quite absent, thus altering the general appearance of the wood very considerably. Moreover, the first few growth-rings from the middle of the bole, may possess a somewhat different appearance from that of later-formed, more mature wood, owing to the smaller size of the elements (*e. g.*, the vessels), and rapid developmental changes from year to year. Heart and sapwood also often differ in colour, density, etc.

In a number of commercial species, such as oak, beech and hazel, one of the most salient diagnostic features is the occurrence of large medullary rays (storage tissue), conspicuous to the naked eye. As the distribution of the latter is sometimes irregular, a small sample may easily be chosen lacking any such rays. Thus, to an unpractised eye, a sample of common oak wood devoid of large rays can be mistaken for that of sweet chestnut, or a narrow-ringed piece of alder wood, lacking the large rays, for that of the pear or apple.

Hence, it will be realised that the selection of a truly representative sample is of vital importance; and where such samples are not obtainable, any identification that may be arrived at will be correspondingly unreliable.

In the following descriptions, it should be understood, therefore, that heartwood samples of fair size from the main stem, of medium age and ring width, are indicated. And it should be added that a portion of at least one whole growth ring (where present) should be included in the thickness of the sample block, and preferably two or three.

Preparation of Material for Sectioning

(a) **Softening.**—Wood samples will rarely be encountered sufficiently soft in their natural condition for immediate sectioning in a microtome; the majority of woods, even soft woods such as are produced by the Conifers, being hard enough to turn the edge of a sharp steel knife. Hence, where it is decided that microscopic sections will be needed for identification purposes,[1] the sample block must first be softened. This may be effected as follows:—

[1] The alternative, or rough "field" method, consists simply in cutting off clean transverse and longitudinal-tangential surfaces, and cleaving (not cutting) a small radial surface, for critical examination with a hand-lens. For further details, see *Wood Charcoals*, pp. 457 *et seq.*

(1) Boil the sample in tap water, under a wire gauze or tin funnel, for 5–12 hrs., until completely water-logged.

(2) Transfer from water to diluted hydrofluoric acid, kept in gutta-percha, stoppered-jars, in which solution the samples should remain for a period of 1–4 weeks, according to relative hardness. The strength of the hydrofluoric acid may also be varied according to the wood being "very hard," "hard," "medium," or "soft." For instance, a wood such as oak (rated as "hard" to "medium") may cut satisfactorily after only one or two weeks in a 50% hydrofluoric solution in water; whereas a "very hard" wood such as *Lignum vitae* may require four weeks or so in almost concentrated hydrofluoric acid.

(3) Thoroughly wash out all traces of acid from the blocks in running tap water—not less than 24 hrs.

(4) If the blocks are not immediately required for use, store in a mixture of 50% glycerine and 50% methyl alcohol. This solution appears to have a further action upon the wood, rendering it more mellow for cutting. On the other hand, samples left in this solution for more than a few months or a year may ultimately become too soft. Alcohol also has a deleterious effect upon the staining properties of plant tissues.

Earlier workers often tended to over-estimate the necessity of hydrofluoric acid treatment and subsequent storage in glycerine-alcohol. Most coniferous and other soft woods can be sectioned quite well in a heavy sledge microtome without anything further than 8 to 10 hrs. boiling in water; while woods of medium hardness (*e. g.*, oak) can usually be cut after 12 hrs. boiling in water, followed by a limited storage in glycerine-alcohol. And though the cutting knife must be expected to blunt more quickly, time lost at grinding and stropping will be amply repaid by the elimination of the hydrofluoric acid treatment, when quick results are required.

Storage of wood blocks in glycerine-alcohol is most conveniently effected by the use of small glass specimen tubes, large enough to take two or three blocks. Duplicate samples are desirable.

The softening of wood samples in a 12% solution of cellulose acetate[1] in acetone, after evacuation in water and two hours soaking in acetone, should also be cited. The blocks are left in the acetate

[1] Cellulose acetate as prepared by the Société Chemique des Usines du Rhone is recommended by various workers in preference to other brands.

solution for a period varying from two days to a month according to their relative hardness, after which they will usually cut well. But many workers prefer the hydrofluoric acid method as being more certain.

(b) **Sectioning.**—The next step in the preparation of material for examination is microtoming.

First, the sample should be cut square. It is then ready to be clamped in the Naples holder commonly provided with good sledge microtomes.[1] The final orientation of the block is allowed for by the Naples holder, which can be obtained to take objects up to about 2 in. in diameter. A flat-ground knife[2] should be used for normal wood, the blade and the block being kept flooded with methylated spirit whilst cutting is in progress, and the section very gently held in place, to prevent it rolling up, by means of a large camel's hair brush. On the standard sledge type of microtome, which may weigh anything from 20–80 lbs., good sections can be obtained from suitably prepared hard wood blocks at thicknesses from 2–30 μ. On the older models the block was raised by hand, using a ratchet device, but on more recent instruments the same thing is performed automatically, to any given thickness.

The most generally useful thickness for wood sections is 10–15 μ., sections being cut in three directions, as noted above.

It will usually be found best to cut the transverse section first, the tangential-longitudinal second, and the radial-longitudinal last; the transverse being the most important microscopically, and desirable in as large a size as possible.

In sectioning, the grain of the wood should lie parallel to the long axis of the microtome, with the cutting-knife skewed at about 45° to the long axis of the latter, and canted slightly downwards towards the block in accordance with the hardness of the wood.

As the sections are cut, they may be gently removed from the knife to a bath of methylated spirit, or water, according to subsequent staining treatment—the tip of a finger being the best way of doing this.

Sections can, of course, also be cut with a hand razor, but are seldom obtainable in large enough sizes or of sufficiently uniform thickness, by this method, for thorough examination.

[1] Those supplied by Messrs Reichert of Vienna are considered to be satisfactory, and may be obtained at prices ranging between £15 and £40—the smallest model being suitable for most ordinary purposes.

[2] Those by Messrs Jung of Heidelberg are recommended.

(c) **Staining.**—After sectioning comes staining; and it is here that there is most scope for variety of technique. A great number of common dyes stain both cellulosic and lignified woody tissues. The essential characters of a good tissue stain are, first, that it shall be selective—not staining other tissues or materials of different chemical composition; second, that it shall be translucent, yet distinct to the eye; third, that it shall have good photographic properties—either alone or when combined with suitable selective colour filters; fourth, that it shall "take" well and not fade; fifth, that it shall contrast suitably with a selected counterstain; sixth, that it shall be easily obtainable of a consistent quality; seventh, that it shall hold fast during the process of dehydration with alcohol, nor be soluble in the solvent used in the mounting medium. These specifications naturally reduce the total number of suitable dyes available.

The most generally useful combination for routine work is, perhaps, Haidenhain's iron-haematoxylin followed by safranin; the former stains any cellulosic structures and cell contents a prominent blue-black, the latter dying the lignified cell walls a deep, clear non-actinic red. Sections prepared in these colours give admirable images on photomicrographs—especially if panchromatic plates are used, without the employment of correcting colour filters. For visual work, however, a pale spectral-green filter renders the red more restful to the eyes.

As the foregoing combination has been widely accepted in wood structural laboratory practice, it will be well to describe it more fully. Assuming then, that the material has not had its staining properties spoiled by some previous chemical treatment or undue storage in alcohol, the following procedure may be adopted subsequent to microtoming.

1. Leave sections to soak in tap water for 2–5 mins.

2. Replace water with a 3% aq. solution of iron-alum for 5–10 minutes.

3. Thoroughly remove all traces of iron-alum by several changes of water. (Otherwise, precipitation of the haematoxylin occurs in next step.)

4. Stain from 2–5 mins. in a dilute solution of Haidenhain's haematoxylin in water, until sections are dark slate-grey.

5. Wash thoroughly in several changes of water to remove excess of stain. (Over-staining can be rectified by immersing again for a

few moments in the dilute iron-alum solution, until the requisite density has been obtained, then washing thoroughly in water.)

6. Replace water by 50% alcohol.

7. Add about a dozen drops of the strong safranin solution[1] to the last bath, sufficient to give a clear light red. Stir well, and leave dish covered for 12–48 hrs., until the sections are sufficiently densely stained.

8. Decant stain, and wash sections in several changes of methylated spirit to remove excess. (Any further excess can be removed by treatment with acid alcohol[2] and further washing in plain alcohol, but it is desirable not to over-stain the sections in the first instance. For the latter reason, long staining in dilute safranin is always safer than shorter treatment with strong safranin.)

9. Wash the sections in three changes of absolute alcohol. (A small quantity each time will suffice.)

10. Decant alcohol and cover with pure xylol. (Any other clearing oil, such as clove oil or cedar oil will do well, and the latter have the advantage over xylol that they do not cause the sections to harden and curl so much. On the other hand, xylol is far cheaper, is the usual solvent for Canada balsam, as mounting medium, and proves quite satisfactory if not left too long.)

(d) **Mounting.**—The sections are now ready to mount in some medium such as Canada balsam, Euparal, Camsal balsam, or Gurr's medium; only in the latter case, xylol should not be used as clearing agent, as it curdles the medium. Most microscopists find Canada balsam perfectly good. Preferably, it should be used rather concentrated, and applied hot, slightly in excess. In this way, subsequent shrinkage of the medium with evaporation of the solvent is reduced, the excess medium is drawn under the cover-slip when incubated (*vide infra*), and so the formation of large air gaps beneath the cover glass is avoided. It is customary to mount the transverse, tangential and radial sections on one slide.

After mounting, the slides should be left for several days in an open rack (names or numbers may be marked temporarily in grease pencil), to permit some of the solvent to evaporate off. They may then be pressed down with small spring clothes-clips, two to each slide; care must be taken to insert, first, a small piece of blotting-

[1] This is best made up as follows: 1 % solution of alcohol-soluble safranin in 95 % alcohol, mixed with an equal volume of 1 % aqueous safranin in water.

[2] Methylated spirit *plus* a few drops of hydrochloric acid.

paper, then a thin flat slip of wood,[1] between the clips and the cover glass. In this condition, the preparations are placed horizontally in an oven heated to 60° C. for 6–12 hrs., after which they are taken out and left to cool and harden, without unclipping, for several hours more. Finally, the clips, etc. are gently removed, the slides cleaned round with xylol, and labelled. They are then ready for use, though it is not wise to employ these fresh-made preparations on a hot stage, such as that of a micro-projector for instance, for hardening only proceeds slowly, with lapse of time.

If only a temporary or semi-permanent mount is required, the entire process, subsequent to sectioning, may be both simplified and accelerated by mounting the unstained section direct from water in glycerin jelly mixed with a little stain, such as aniline or methylene blue, or such other more permanent colour as may be preferred. The cover glass should subsequently be carefully cleaned round, and ringed with an airtight varnish, wax, or cement.[2] In temperate climates preparations of this sort will keep for many years, but some skill is needed to avoid the inclusion of small air bubbles in the jelly.

(e) **Alternative Staining Methods.**—The standard staining technique, with Haidenhain's haematoxylin followed by safranin, has been described. The former, however, being essentially, a precipitation stain and rather opaque, is not altogether reliable, unless carefully employed and interpreted, where the discrimination of minute details is at stake. Safranin again, though convenient, is by no means an ideal stain. One or two other possible combinations should, therefore, be mentioned.

For instance, an old, tried favourite with botanists, and one which the writer has also found to give excellent results for photomicrography—especially with suitable selective colour filters—is the combination of gentian violet with orange G. Another pretty differential method, also well known, is safranin followed by lichtgrün.[3] In the first instance, the lignified tissues stain violet to blue (a certain amount of dual staining being effected by the methyl violet dye alone, as with Biebrich scarlet—*vide infra*), and the soft tissues orange. In the second instance, the lignified tissues stain

[1] Three-ply board, cut into pieces of about 2 × 1 inch size, does well.

[2] Paraffin wax, beeswax, gold size, or any commercial ringing-cement will do. *N.B.* The slide and cover-slip must be clean and dry, or the glycerin will work through.

[3] Grübler's stains are recommended, though other reliable makes are now available.

red and the remainder a fine, clear grass green. In fact, for sheer differentiation, lichtgrün counterposed against a scarlet or crimson dye gives an insurpassable contrast. Unfortunately, however, it tends to fade in the light, and there is a common complaint that lichtgrün also tends to replace the safranin from lignified tissues.[1] A number of trails, checked by suitable microchemical tests on control material, however, have convinced me that this is not necessarily so. Provided that the material is first stained thoroughly in diluted safranin or conc. Biebrich scarlet (*vide infra*), and no acid alcohol used intermediately, I do not find that there is any "poaching" on the part of lichtgrün. It is conceivable, therefore, that, when replacement has been suspected, either the technique has not been properly effected, or one has erroneously presumed as to the microchemical composition of the cell walls.[2]

As remarked above, the staining property of tissues may readily be spoiled by undue storage—in alcohol for instance. This certainly applies to safranin and Biebrich scarlet. Experiments have also shown that the staining reaction of a given substance or tissue may be reversed by suitable chemical treatment, changing the sign of the electrical surface charges on the molecules.[3] Also the actual size of granules may determine their staining property. There is thus a distinct danger in trusting implicitly to staining reactions as a sign of chemical constitution, though fresh, untreated material may usually be relied upon to produce relatively constant resultant colours in answer to a standard technique. Yet, even here, the frequent presence of absorbed substances in the cell walls may modify or mask an expected reaction.

When gentian violet is followed by orange G, the following procedure may be adhered to in staining wood sections.

1. Stain sections either in a saturated (filtered) solution of gentian violet in 50% alcohol and water, for 15–20 mins., or, preferably, stain overnight in a more dilute solution. (*N.B.* It is best rather to overstain, as gentian violet washes out readily in the subsequent treatment.)

[1] *Vide* B. Lee's "*Microtomist's Vade Mecum*" (ninth edn.), art. 324, pp. 185–6.

[2] Actual tests carried out upon a variety of plant species make it amply clear that the distribution and frequent intergrading of such substances as lignin, cellulose, pectin, cutin, etc., is by no means as uniform and simple as some of the earlier text-books suggested. For instance, see "*Distribution of Lignin in Wood*," by G. J. Ritter, in *J. Ind. Eng. Chem.*, 1925, **17**, 1194. (Ritter shows that two types of lignin exist in wood, and that the middle lamella, or primary wall, is not rich in pectin, as has been asserted.)

[3] *Vide*. "*Fixierung, Färbung und Bau des Protoplasmas*," by Alfred Fischer. Jena: Gustav Fischer, 1899. Also "*The Macrophages of Mammals*," (p. 255), by H. M. Evans, in *Amer. J. Physiol.*, **49**, 243. Also the work of Paul Ehrlich.

2. Wash rapidly in two changes of 95% alcohol in order to remove excess of stain, and start dehydration.

3. Follow up with two rapid washings of absolute alcohol, to dehydrate, leaving the sections still rather overstained.

4. Differentiate and counterstain with a saturated soln. of orange G in clove oil, until lignified elements are of desired density of violet. (*N.B.* As gentian violet comes out rapidly in the clove oil, employ a strong solution of orange, else the latter will understain the softer tissues.)

5. Finally, fix the stains by immersing in xylol.

Where safranin is followed by lichtgrün, the same hurry need not be observed, as safranin does not wash out to the same extent as violet in alcohol or clove oil. Otherwise, the various steps are the same as before. For preference, stain slowly and heavily in a somewhat diluted safranin for at least twenty-four hours, wash out superficial excess in methylated spirit, dehydrate in absolute alcohol, and counterstain with lichtgrün in clove oil. A fresh, saturated solution of the green dye should be used, so that immersion in the oil need not be unduly prolonged; nor should the sections first be overstained in the safranin.

To avoid displacement of the safranin, light preliminary mordanting with potassium permanganate (see below) is sometimes advisable.

In addition to the foregoing, the undermentioned permanent stains may be employed in wood-structural and other botanical work, where differential staining of lignified and cellulosic tissues is desired.

LIGNIN STAINS

Ammoniacal fuchsin	Should be made up very strong.[1] Use after clearing with Eau-de-Javelle[2] and washing thoroughly in water. Staining is aided by a small piece of copper wire. Subsequently, wash very long in methylated spirit in open dish. Clear in xylol for four hours.
Acid fuchsin	Used in 1% aq. solution.
Methyl green Methyl blue. Methyl violet	All powerful stains for general work. May be used in alcoholic solution or in glycerin jelly, used as mountant.
Eosin	Alcoholic soln. Not very reliable, but very pretty followed by haematoxylin or lichtgrün.

[1] Aq. basic fuchsin is added to conc. ammonia, until the mixture is straw-coloured.
[2] Potassium hypochlorite.

LIGNIN STAINS.—(*Continued*)

Erythrosin	Used in 1% aq. solution in place of eosin. It is a rapid stain.
Cyanin	Dissolve in 95% alcohol and add equal vol. of water.
Magdala red	1% solution in alcohol.
Congo red	1% aq. solution.
Iodine green	Use in alcoholic solution. Be careful to wash out from cellulose.

CELLULOSE STAINS

Haematoxylin (or Haematin)	Several varieties of this ("logwood") dye are available, besides Ehrlich's acid and Haidenhain's *iron* haematoxylin. Iron and aluminium are the commonest mordants, and the solutions always require "ripening."[1] Haematin is a good plasma and cellulose stain.
Bismark brown	Alcoholic solution used. Not so precise as haematoxylin.
Malachite green: Acid green	Aq. solutions 1%.
Aniline blue	Strong solution in methylated spirit.

Note.—Plenty of powerful dyes may also be had which will stain all tissues indiscriminately (*e. g.*, brilliant acid blue), but the above named are among the few found suitable as counterstains, to dye the cellulose and lignin differentially.

[1] This may be done artificially by means of hydrogen peroxide.

Yet another extremely valuable lignin stain not mentioned above is Biebrich scarlet; a stain of great translucency and good photographic quality (orange-scarlet), though not always easy to use.

Biebrich scarlet, which is prepared from a diazo compound of acid yellow combined with β-napthol, was discovered by Nietzki in 1879. It is, therefore, a double dye, giving, in fact, a range of colours shading from light orange to almost crimson. In purely botanical work the writer has found it invaluable for differentiating betwixt the various lignified, semi-lignified, suberised and cutinised portions of the plant; it is superior to gentian violet in this respect. Biebrich scarlet will also be found valuable in the preparation of wood sections, when used in conjunction with a differential stain such as lichtgrün, or acid haematoxylin followed by dilute ammonia.

As Biebrich scarlet has not yet come into very general botanical use, the following details are of interest.[1] Make a saturated solution of the dye in a mixture of glycerol, 2%, methylated spirit, 8%, and distilled water, 90%. (*N.B.* It forms a turbid solution in water, and is only moderately soluble in alcohol.) Stain sections

[1] *Vide*, however, S. G. Scott's Standard Haematoxylin and Biebrich Scarlet for Chromophility (*J. Path. and Bact.*, 1912, 16) and other cytological uses.

for at least 48 hrs. in this solution, wash quickly in clean methylated spirit, followed by two changes of absolute alcohol, and counter-stain in a solution of lichtgrün in clove oil. Alternatively, stain in Ehrlich's acid haematoxylen, wash out excess with methylated spirit, make the haematoxylin lake blue with ammonia, then stain in Biebrich scarlet as before. Very beautiful preparations—never overstained—can be obtained in this way with care.

(f) **Mordanting.**—Material which has been left unduly long in alcohol, or otherwise subjected to drastic chemical treatment, may refuse to stain well with dyes such as safranin, gentian violet, Biebrich scarlet, rubin, vesuvin, etc., without mordanting. For the latter purpose dilute potassium permanganate is usually satisfactory—though even after this powerful mordant[1] I have known wood sections (of common oak for example) to remain intractible.

The accepted method for permanganate mordanting, (due to Henneguy: *J. de l'Anat. et de la Physiol.*, 1891, **27**, 397), is as follows:—

First, treat sections for about five minutes in a 1% solution of potassium permanganate. Then wash well with water, and finally stain with safranin (or other dye, as chosen) for about half the ordinary time. Excess of stain—especially if rubin be used—can only be removed by prolonged soaking in clove oil.

Examination of the Sections.—Preparation complete, the sections may then be examined with a moderate power of a compound microscope, assisted (when a larger field is desired) by dissecting and low power binocular microscopes. As a rule, a student-type compound microscope, giving as large and flat fields as possible, with magnifications ranging from 50 to 500 × linear, will be found ample for all wood-structural work and identification. Rarely a 1/12th. oil-immersion objective and a somewhat higher magnification may be employed with advantage for the more minute examination of the pitting and lamination of the cell wall; but the latter, more academical aspects of Wood Anatomy we may pass over, since the diagnostic details commonly used for identification are observable with quite moderate optical equipment.

Indeed, a good low-power binocular microscope, magnifying from 10–40 × linear, represents one of the most valuable assets for this work . . . A broad view of the woody structures *en masse* always

[1] *Vide.* B. Lee (*loc. cit.*).

remains a perequisite, whatever detailed examination may follow after; hence, merely a small hand-lens (mag. 8–10 × lin.) is commonly employed.

All three sections, transverse, tangential longitudinal, and radial longitudinal, may be needed; but the most typical and easily remembered view of a wood is that of the transverse section. The latter should, therefore, always be examined first.

The grouping and arrangement of the "*pores*" (vessels), *fibres, tracheids*, "*soft tissue*" (parenchyma), vertical *resin ducts* and *medullary rays*, as well as such special features or abnormalities as *pith flecks, stone cells, intraxylary phloem*, etc., may all be seen to advantage on the transverse cut, or "end grain," as joiners term it. (*N.B.* The cut must, of course, be very clean, and moistening often helps to show up parenchyma, resin ducts, etc.)

The presence of spiral thickenings, pitting and end perforations of the vessels, the height and shape of the rays and ray cells, the presence of scattered parenchyma, horizontal resin ducts, crystal cells, etc., on the other hand, should be looked for upon the tangential and radial longitudinal sections.

In fact, one may say that, where woody species possess a very similar general anatomical structure—as in the case of the *Pyrus* genus, or the willows and poplars, or many coniferous genera—then one must depend upon longitudinal, rather than transverse, sections for final identification. Unfortunately, however, there are many genera in which intraspecific variability of structure is as great as any interspecific distinctions. In such instances, the best that the wood anatomist may do is to designate the genus. Thus, in identifying small fragments of European charcoal, for instance, the discrete species of poplar and willow are simply designated "*Populus sp.*," or "*Salix sp.*"; maple and sycamore are given as "*Acer sp.*"; apple, pear, beam, wild service, true service, medlar, and rowan (mountain ash) as "*Pyrus sp.*" For to profess to diagnose the particular species would sometimes be little better than pure speculation.

But given fair material and experience of a given genus, even these closely similar species may occasionally be segregated.

Yet, apart from such difficult examples, it needs only a little experience to enable anyone provided with a sharp knife, or microtome, and a low-power microscope to distinguish with certainty between common oak and sweet chestnut, hazel and hornbeam,

elm and laburnum, or apple and hawthorn, let us say. Yet to the uninitiated or the unaided eye these pairs of woods may appear structurally similar.[1]

DECAYED WOODS

Choice of Material.—Wherever the better preserved and tougher portions of the original sample are also representative of the whole, there is little difficulty. But more frequently the whole piece is rotten. In sampling, as for normal wood, blocks should be cut about 0.5 inch square on the transverse section by about 1.0 inch long; though smaller pieces may sometimes be used, if unavoidable. The blocks should, of course, be cut square to the grain.

In certain instances, when the wood is very dry and powdery, it will be found possible to break the original fragment across between the fingers, rather than by cutting or sawing. But longitudinal shaping should be done with a sharp knife. Soft and damp specimens, however, may best be cut transversely with a very sharp razor, care being taken to avoid crushing the tissue unduly.

Preparation of Material.—Rotted woods may either be fit to section immediately, without further treatment (as when soft and damp), or they may be so brittle and crumbling that impregnation with a waxy, gelatinous, or glutinous medium is first necessary. It is desirable, in this respect, as far as possible to match the medium to the relative hardness of the tissues. Thus, a very soft wet sample should be embedded in paraffin wax, and a brittle, dry, crumbling specimen in Celloidin, or collodion, (cellulose nitrate). It is assumed that no previous softening either by boiling water, glycerin, or hydrofluoric acid will be needed.

(a) **Paraffin Embedding.**—This method is the same as that employed by cytologists, zoologists and others interested in the sectioning of soft, flexible or delicate tissues.

1. Thoroughly evacuate the samples under the vacuum pump, using a 50% aq. sol. of alcohol as medium.

2. Dehydrate in several changes of methylated spirit, followed by absolute alcohol.

3. Decant alcohol and substitute pure xylol. Add paraffin wax chips to xylol (cold) until no more dissolves.

[1] *Note.*—For further details and examples see the section on *Methods of Identification*, p. 458.

4. Place the bath, in open condition, in a 60° C. oven to evaporate off the xylol. (A small specimen-tube is convenient.)

5. When the paraffin no longer smells of xylol, transfer specimens to another bath of pure melted paraffin wax, leaving several days at 60° C. to impregnate thoroughly.

6. Remove samples with forceps, drop into water to cool and harden for several hours. Then square up ready for sectioning.

Material embedded this way, will usually section well with a slightly hollow-ground knife, a sledge microtome being used, as for normal wood. Or, if the specimen is small, or serial sections are desired, section with a Cambridge rocker or rotary microtome.

The sections should next be glued on to the slide with egg albumen, as for cytological preparations. This is done by, first, smearing the clean side with a drop of the prepared albumen,[1] followed by two or three drops of water from a pipette, next, floating on the selected sections (in wax), and then warming the water gently from below, until the wax and contained sections flatten out without quite melting. Finally, drain off water on filter paper, and carefully but firmly iron down the sections upon the albumen, using a slip of smooth filter paper and the ball of the thumb. Leave the preparation to dry and harden.

Subsequently, the wax matrix may be dissolved off with xylol, and the sections stained either horizontally or vertically—in a staining trough. Completion of mounting is as usual.

On the other hand, when the sections are sufficiently coherent, the wax may be dissolved away and the sections stained in the usual manner without the use of albumen.

Great care, however, will be needed in handling the free sections.

(b) **Celloidin Embedding.**—Perhaps the best method of embedding crumbly or rather dry and brittle samples is in collodion—now sold commercially as "Celloidin."[2]

One commonly accepted procedure, described below, involves the use of a series of solutions of Celloidin in a mixture of equal volumes of absolute alcohol and ether, ranging from 2%–16%, in steps of 2%. These, therefore, should be made up first, and kept in stock bottles. Another requisite is a number of small, but strong,

[1] Mayer's albumen consists of white of egg, 50 c.c.; glycerin, 50 c.c.; and sodium salicylate, 1 grm. Shake well together and filter slowly.

[2] Closely related substances are known as "*Celloidinum inelasticum*," "*Photoxylin*" and "*Collaxylin*," respectively.

wide-necked, screw-topped jars, to contain the samples while infiltrating. Wired-on corks will do in place of screw tops, but the bottles must be air-tight and able to stand some internal pressure.

1. Place material in methylated spirit, and remove all air from the tissues under the vacuum pump.

2. Substitute fresh methylated spirit for old, and leave over night.

3. Complete dehydration, using successive changes of absolute alcohol, and stirring occasionally. (Time taken depends upon the relative porosity of the tissues.)

4. Transfer samples to sealed pressure-jar containing 2% sol. of Celloidin in ether and alcohol. Leave in oven at 40° C. for 24 hrs. to two days, according to relative porosity.

5. Change material to a second jar containing 4% Celloidin[1] . . . and so on throughout the series, up to the 16% solution, giving 24–48 hrs. in each solution.

6. Finally, pick out samples from Celloidin while still warm, and drop into a mixture of equal parts of ether and chloroform. In this bath the celloidin will gradually harden and can subsequently be shaped up for microtoming as usual.

After sectioning, celloidin preparations can, of course, be cleared and stained. But the writer has found that this is usually unsafe, unless the albumen method be employed, as the structures are in danger of blurring by slight misplacement or fragmentation. A surer way is to mount the loose sections, infiltrated with Celloidin as they are, either in glycerin jelly or in Gurr's mounting medium; which last appears to possess the property of slowly dissolving away the Celloidin and clearing the section *in situ*.

Material embedded and prepared this way can usually be identified, even though decay or compression had previously rendered it almost amorphous to the unaided eye. Examination of the sections will, of course, follow the same lines as with sections of normal wood or charcoal.

COMPRESSED WOODS

Compression of archaeological and excavated specimens will more often than not be found associated with decay; in general, the foregoing technique is applicable. But where compression is so severe (especially tangentially) as to blot out the greater part

[1] The jars should be allowed to cool before unstopping.

of the fine details, it will sometimes be possible to swell and partly reform the cells by prolonged soaking in hot water. But note that, with delicate tissues, actual boiling is to be avoided.

Samples can often be made more recognisable in this way, especially if the structures are still entire and the compression relatively recent.

Material which bas been excavated from wet situations and suddenly exposed to light and air sometimes shows an excessive shrinkage, technically termed "collapse." This may sometimes be rectified in the same way as compression, provided the wood is not allowed to dry out a second time.

Subsequent treatment will be as for normal or decayed woods, according to circumstances.

WOOD CHARCOALS

Although the sectioning and identification of wood charcoals might at first sight appear, and has by many been believed, to be extremely difficult or impossible, in actual fact, it is relatively simple.

Charcoals of many woods of coarse texture, or familiar and unmistakable structure, such as common oak, ash, and elm, may be identified from the transverse section, when simply snapped cleanly across the grain between the fingers, and examined with a hand-lens or low-power binocular microscope. This method will suffice in a large proportion of instances, where the investigator knows the woods of the country well, or possesses a reliable "key." (*Vide infra.*) In other instances, microscopic sections will be needed.

(a) **Surface Painting.**—Really good sections of decayed or brittle woods and charcoals can only be obtained by Celloidin embedding; but where press of time or economy forbids the longer process, simple surface painting of the squared block with a 5% solution of Celloidin before each cut will often permit of rough sections being made. For although these may have a comparatively rough appearance compared with a cleanly broken surface, examined under a binocular microscope, they have the advantage of being examinable by transmitted light.

Two difficulties do, however, exist, in identifying charcoals: namely, first, that they are often too crumbly to allow of treatment with alcohol and ether, evacuation, etc., owing to excessive dampness, or other conditions of preservation, and second, that certain

minute structures may not be sufficiently well preserved, or sufficiently clear under the microscope, owing to opacity, to permit of proper diagnosis. The writer has, nevertheless, found that what is unobservable in one section, can often be seen in another. Hence, charcoals can usually be identified as well as normal woods.

(c) **Embedding in Synthetic Resin.**—Hard and "glassy" charcoals may be embedded in a synthetic resin, in *lieu* of Celloidin, either by complete impregnation or by surface painting. A resin found suitable[1] for this process consists of 6 mols. of formaldehyde, 3 mols. of phenol, and 3 mols. of meta-cresol. This, when the charcoal is thoroughly impregnated and heated, hardens into a glassy matrix, which can then be sectioned with a steel and diamond wheel, according to the ordinary petrological method, and the sections subsequently ground down on a slide to the required thickness. About four hours of initial heating at 45°, followed by further heating at a temperature rising to 100°, until the resin is sufficiently hard, has been found to be suitable.

The preparation of good thin sections by this method, however, requires too much time and labour to make it competitive with Celloidin embedding.

METHODS OF IDENTIFICATION

A. **Anatomical Methods.**—Microscopic examination of a few cross sections of different woods will at once make it clear that woods vary considerably in anatomical structure, though upon longitudinal sections this is not always so evident. The transverse section, therefore, is the first aspect of a wood required in identification.

Any one with a little experience of a small group of woods such as those indigenous to Europe should soon be able to determine at a glance the genus (of the coarser grained species at least) from the cross section alone. Moreover, this he will be able to do with far greater certainty than the everyday forester, joiner or timber merchant, who merely recognises, or thinks that he recognises, them by their less reliable macroscopic appearance, weight, colour, etc. For the former purpose, as we have already seen, a hand-lens and a sharp knife may be sufficient. There is, however, a number of the finer textured woods that cannot be so readily distinguished: willow

[1] Information derived from experiments on behalf of the Forest Products Research Lab., Eng., carried out by the Building Research Station, D. S. I. R., 1928. (Private enquiry.)

and poplar for example. These require good sections and a compound microscope, in order to determine the presence or absence of, form and frequency of certain more minute structures invisible with a hand-lens.

Both general and detailed features must next be considered.

(a) **General Features.**—The principal features useful in making generic and (though less commonly) specific diagnoses are as follows:

Pores.—The relative size, disposition, frequency and configuration of the conducting vessels, or "pores," as seen in transverse section.

Rays.—The relative size, disposition and frequency of the medullary, storage, or "pith" rays, as seen in transverse and tangential longitudinal sections.

Fibres.—The type, grouping and disposition of the wood fibres, sclerenchyma or "mechanical tissue," as seen in transverse and radial longitudinal sections.

Tracheids.—The type and disposition of the tracheids and/or fibre-tracheids, as seen in transverse- and radial-longitudinal sections.

Parenchyma.—The relative proportion, distribution and configuration of the vertical storage parenchyma, or "soft tissue," as seen on transverse, and radial longitudinal sections.

Resin Ducts.—The occurrence, relative frequency and disposition of the resin canals, where present, as seen in transverse and tangential-longitudinal sections.

All these features may just be made out with a good hand-lens or low-power binocular microscope upon a critically illuminated and cleanly cut surface in normal wood, though hardwood tracheids and vertical parenchyma are frequently impossible to distinguish either in charcoals or rotted wood without good thin sections.

As has been indicated, the size of the rays and pores is clearly variable, though the fibres, tracheids and parenchyma vary more obviously in amount than in cellular dimensions, which are microscopical.

"Hardwoods" and "Softwoods."—In commerce, timbers are conveniently classed as "hardwoods" and "softwoods" respectively, according to whether they are derived from coniferous (*i. e.*, *gymnospermous*) or broad-leaved (*i. e.*, *angiospermous*) trees. As for the great group of palm-like, monocotyledonous angiosperms, they are not of sufficient timber value often to trouble the wood anatomist, though extremely important as a source of comestibles.

The terms "hardwood" and "softwood" are sometimes misleading, for there are many commercial species among the broad-leaved genera, such as certain balsas, liriodendrons, willows and poplars, considerably lighter and softer than many of the so-called "softwoods." In short, relative density and hardness depend almost directly upon relative porosity, and many "hardwoods" are highly porous. (See also below: *Specific Gravity.*)

Anatomically, however, the distinction between the two classes of timber is unfailing, consisting as it does in the complete absence of true conducting vessels ("pores") and fibres from the coniferous woods, in place of which one encounters a ground mass of fibre-like conducting elements, termed tracheids, with irregularly distributed resin ducts. Resin ducts may or may not be present. Some Gymnosperms have none.

The presence or absence of true "pores," or conducting vessels, therefore, affords an immediate criterion as to the angiospermous or gymnospermous nature of a given wood.

Growth Rings.—Growth rings—usually *annual* growth rings—can be seen (most clearly on the transverse section) in all trees from regions affected by seasonal climatic changes. Evergreen tropical trees, however, normally lack any distinct growth rings, the successive zones of varying density and structure in deciduous and colder climate trees being attributable either to inherent growth rhythms or to cyclic climatic changes directly affecting the metabolic rate.

Hence, the complete absence of distinct growth rings may be taken to mean that the timber in question was derived from an evergreen tropical species. There exist, however, some tropical trees—possibly more recent immigrants—which still persist in a restricted deciduosity, and, accordingly, show growth periods in the wood.

Ring Porosity and Diffuse Porosity.—Another broad diagnostic distinction is the fact that in certain non-tropical hardwood trees the pores[1] are so distinctly larger, or more numerous, at the inner edge of the growth ring as to form a conspicuous annular zone. Such woods are termed ***ring-porous.*** Other species have their pores more uniform in appearance, and decreasing much more gradually

[1] Seen as minute oval-shaped holes, standing out more or less conspicuously against the groundwork of fibres in transverse section.

in size. These are termed *intermediate-porous*. While, in a few, the pores are practically uniform in size and number throughout the wood. The latter are termed *diffuse-porous*.

Thus tropical hardwoods are typically diffuse-porous; though why many temperate woods such as *Acer* and *Cornus spp.* are also almost truly diffuse porous, despite the summer-winter climatic cycle is hard to explain; unless, indeed, we might assume a relatively recent immigration of the latter from more equable regions.

In speaking of "diffuse-porous" and "ring-porous" woods, however, it must be realized that the two extremes of an intergrading series are referred to. Very few woods are truly diffuse-porous, nor are strictly ring-porous woods common, the majority falling intermediately betwixt the two types.

For purposes of classification, therefore, it is convenient to employ the three broad categories already mentioned. Thus, amongst European genera, *Acer*, *Pyrus* and *Carpinus spp.* would be termed diffuse-porous, *Juglans*, *Prunus* and *Lonicera* intermediate-porous, and *Quercus*, *Ulmus* and *Fraxinus* ring-porous. Detailed descriptions, however, should always be added as surety against misinterpretation.

Vessel Size.—In hardwoods the relative size of the pores is of importance;

whether "Large" . . . . *i. e.,*	Conspicuous to the naked eye.[1]
"Moderate"	Fairly easily visible to the naked eye.
"Small"	Only just visible to the naked eye.
"Very small"	Invisible to naked eye, but distinct with hand-lens.[2]
"Minute"	Scarcely visible with hand-lens.

[1] By naked eye is implied normal good sight.
[2] By hand-lens is meant a good low-power lens magnifying about 8 × linear.

By way of example, common oak (*Quercus robur*), ash (*Fraxinus excelsior*), maple (*Acer campestris*), alder (*Alnus glutinosa*), and box (*Buxus sempervirens*) may be taken to represent the five categories.

Vessel Pattern and Grouping.—So far, we have spoken of the distribution and size of the pores. In addition, there are the grouping and configuration, or "pattern" of the vessels, as seen on transverse sections.

In some woods the pores are chiefly solitary; for example, in Dagwood or "dogwood" (*Cornus sanguinea*) and maple (*Acer. Sp.*).

In others—the majority—the pores are in groups of two or more; birch and poplar, for instance, frequently have their pores in small radial groups of two or three (derived from a single cambial initial); while hazel, olive and ivy form longer radial strings, and elder and plum possess pores clustered tangentially as well as radially. All such features may be referred to as *vessel grouping*.

Vessel pattern, on the other hand, is produced by some typical configuration of the pores in general, whether individual, solitary or grouped, so as to form a specific and recognisable pattern upon the cross section.

Patterning may be of several sorts:—

E. g., Radial streamers.	as in common oak.
Oblique streamers	as in sweet chestnut and gorse.
Wavy tangential bands. . . .	either relatively continuous, as in elm and laburnum, or much broken, as in mulberry and false acacia.

Further, such wavy bands may be either several pores broad, as in laburnum, or only one or two pores broad, as in poplar. In the thicker and more continuous type of banding, the pores are generally embedded in parenchyma.

Finally, it should be born in mind that both the typical number of cells in a pore group and the general pattern formed by the cells of the latter portion of a growth-ring are subject to considerable variation. In general, nevertheless, one may say that the pores of maple appear "mostly solitary," that those of sweet chestnut form "loose oblique streamers," and that those of common elm are arranged in "fairly continuous, wavy tangential bands" . . . and so on. Experience, however, is required to appreciate the range of possible variation within a given species.

Thus, it is often maintained that the woods of alder, hazel and hornbeam cannot be discriminated with safety. The writer has observed, however, that typical wood of average growth from each of the three species has a distinctive microscopic appearance on the transverse section; though occasional small or badly grown samples will be met with in which identity may remain uncertain. So too with poplar and willow wood, and many other such groups.

Type and Distribution of Rays.—Of not less utility in identification work than the pores are the medullary rays—seen as streaks of various width running radially (*i. e.*, divergent from a common

growth centre, or else almost parallel, according to the age of the wood) across the transverse face of a sample. The chief function of rays appears to be the storage of food; but they also may contain short tracheids and horizontal resin canals, as in some conifers, and be used for the conduction of nutriment and sap in a radial direction. That is to say, that the rays may possess either a homogeneous or a heterogeneous structure. The detailed structure, however, will be considered separately below. At present we are concerned only with the general appearance and distribution of the rays upon a transverse section.

For convenience in hand-lens work, rays may be described as

"Very broad" . . . . *i. e.*,	very conspicuous to the naked eye.
"Broad"	Conspicuous to naked eye.
"Moderate"	Clearly visible to naked eye.
"Fine"	Scarcely visible to naked eye, but conspicuous with a hand-lens.
"Very fine"	Invisible to naked eye, but distinct with hand-lens.
"Extremely fine"	Scarcely visible with hand-lens.

As examples of these six types, evergreen oak (*Quercus ilex*), hornbeam (*Carpinus betulus*), laburnum (*Laburnum vulgare*), elder (*Sambucus nigra*), birch (*Betula alba*), and horse chestnut (*Aesculus hippocastanum*) may be quoted.

In some woods, such as elm or lime for instance, the rays are all of one type and relatively uniform breadth. In others, such as hazel and oak, they are of two distinct sizes; very broad[1] and extremely fine. In others again, they are of moderate but variable size, as in beech and plane.

The value of the rays in classifying woods is, therefore, at once apparent. Moreover, in addition to the transverse section, longitudinal sections of some woods reveal numerous equally spaced, minute horizontal striations, (many to the inch) known as "*ripple marks*"—especially upon a polished surface.[2] These lines are shown by the microscope to be due to a storied arrangement of the fine rays; and a count the mean number of ripple marks per inch on a longitudinal surface may be employed as a diagnostic character.

[1] Vide for example, "*Woods with Conspicuously Large Rays*," by W. W. Tupper, in *Tropical Woods*, 1927, Vol. II, No. 11, pp. 5–9.
[2] *Vide* for example "*Occurrence of Ripple Marks' in Woods*," by S. J. Record, in *Tropical Woods*, 1927. Vol. II, No. 9, pp. 13–18. Other storied elements are also mentioned, and families in which they occur are listed.

Ripple marks are far more common in tropical than in temperate hardwoods, two common timbers in which they occur being satinwood and mahogany; whereas they are absent from all indigeous European woods.

Fibres.—Among hardwoods, some species (*e. g.*, birch) have a general ground tissue of fibres, in which the pores and rays appear embedded. Other species (*e. g.*, laburnum and common oak) have the fibre matrix dissected into tangential or radial masses by groups of vessels, tracheids and parenchyma (sometimes collectively termed "soft tissue"). Such formations being conspicuous and easily memorized, are helpful in identification. Thus, in common oak the fibres typically form radial tongues, pointed at the inner end, which are banded tangentially by semi-diffuse parenchyma, and alternate with zones of small ("summer") vessels and tracheids.

Parenchyma.—The vertically placed wood parenchyma, or storage tissue, consists of small iso-diametric, brick-shaped elements, usually conspicuous by their thin walls and abundance of cell contents.[1] Vertical parenchyma may be classed as

"Diffuse" .... *i. e.*,	scattered promiscuously, mostly in solitary vertical lines, throughout the wood.
"Terminal"	Forming a line, or band, at the junction of one growth-ring with the next.
"Banded"	In tangential lines or bands, sometimes continuous, sometimes broken and roughly parallel to the margins of the growth-rings.
"Vasicentric"	Surrounding the pores, or vessels and often joining them together tangentially, or obliquely, into broken or continuous bands. (Also termed *metatracheal.*)

A slightly moistened end-grain view is best for the detection of parenchyma; but it may easily be overlooked upon a poorly prepared surface, or when present only in small amount. Either by reflected or transmitted light parenchyma shows up lighter than the fibres surrounding it. A compound microscope and good sections are necessary for exact determination.

Tracheids.—In hardwoods, tracheids are commonly found in association with the conducting vessels, and being often indistinguishable, except under the compound microscope, are usually classed together with the pores and parenchyma as "soft tissue."

In coniferous woods, on the other hand, the body of the timber, other than that represented by the rays, resin ducts and parenchyma

[1] Gummy, oily and crystalline deposits, if observed in these cells, should be noted.

(where present), is composed of vertical tracheids. For conifers appear to have made shift to do without vessels and fibres, which may, in fact, be looked upon as specialized tracheids.

The main diagnostic value of the tracheids in conifers consists in their variability as to diameter and thickness of wall. For, whereas, in some softwoods these two features remain relatively constant throughout the growth-ring, thus making the general texture homogeneous (*e. g.*, cypress), in others (*e. g.*, larch, Douglas fir and cedar) the tracheid lumina decrease markedly in size, while the walls increase in thickness, toward the end of the season's growth. A much denser, harder zone of late-formed wood is thereby produced, and the resulting texture may be termed heterogeneous. These two zones are known respectively as "*springwood*" and "*summerwood*" in conifers—corresponding to the more porous and less porous (*i. e.*, denser) zones also encountered in hardwoods.

Apart from the vertical tracheids, short tracheids are also sometimes found in the rays; *e. g.*, in *Pinus spp.*:—a good diagnostic feature.

Resin Ducts.—The occurrence of resin canals is limited to softwoods in which, though of sporadic nature, they are of specific value. Resin ducts are minute tubular structures formed from hollow strands of parenchymatous, resin-secreting, tissue, and may be found either vertically or horizontally (in the rays), or both. Thus larch (*Larix sp.*) and spruce fir (*Picea sp.*) possess small vertical resin ducts. In larch they frequently occur in tangential groups of two or three, and are especially common at the margins of the growth-rings. By way of contrast, in Scots pine (*Pinus sylvestris*) the vertical resin ducts are more conspicuous, usually solitary, but often occurring in tangential lines.

In juniper and cypress, on the other hand, there are no resin ducts, while both in *Pinus* and *Larix spp.* they occur horizontally as well as vertically.

(b) **Special Features.**—We have already seen that the foregoing features, while often sufficing to separate the woods of a given region into a number of rough groups, are not always specific enough to permit of final diagnosis. Thus, the woods of alder and hornbeam so closely resemble one another that the only sure distinction depends upon the presence of what are termed "scalariform" end-perforations to the vessels of the alder, whereas in hornbeam the corresponding perforations are "simple." (*Vide* pp. 498, 499.) There is also a slight

difference in the width of the small rays, and in the relative frequency of the large rays in these two woods. Poplar and willow, again, are often indistinguishable, except that, with a compound microscope, one may observe that the former possesses "homogeneous" and the latter "heterogeneous" rays . . . These and other minute, specific distinctions will next be described.

Spiral Thickenings.—Spirally arranged thickenings may occur upon the inner faces of the vessels, in hardwoods, or of the tracheids, in softwoods. Their presence or absence may be accepted as a specific character. For instance, spiral thickenings are present in the wood of yew (*Taxus baccata*), though not in that of cypress (*Cupressus macrocarpa*), and in larch (*Larix europea*), though not in spruce fir (*Picea excelsa*)—a critical distinction between these otherwise closely similar pairs of woods.

Spiral thickenings may be described as resembling the spiral wire occasionally seen wound round high-pressure rubber hose-pipes; only they are on the inside instead of the outside of the tubes.

Ray Type.—The medullary rays have already been classed as varying from "very broad" to "extremely fine." Good sections, however, and a higher magnification permit of the following further classification of ray types.

"Simple, uniseriate" . . . . *i. e.*,	rays of variable height, but typically only one cell broad.
"Diffuse"	Rays of variable width, from one to many cells, but usually of fairly constant height.
"Aggregate"	An appearance as of occasional large rays; in fact, consisting of many smaller rays running in parallel, very close together.
"Compound"	Occasional very large rays formed by the actual fusion of a number of smaller rays.

Examples of these four types may be seen in sweet chestnut (*Castanea sativa*), plane (*Platanus orientalis*), hazel (*Corylus avellana*) and oak (*Quercus robur*).

It should, however, be borne in mind that these four types to some extent intergrade. Moreover, woods containing either compound or aggregate rays will also be found to contain a large proportion of small rays (*e. g.*, uniseriate in oak, multiseriate in beech), from which the larger rays are supposed to be derived by an evolutionary process.

Cell Wall Pitting.—Generally speaking, the type of pit found connecting one cell with another both depends upon and helps to

define the class of cell to which it belongs. On their vertical walls the conducting vessels of hardwoods, for instance, have numerous minute, round or slit-shaped, "bordered" (*i. e.*, funnel-shaped) pits; so too have the tracheids of conifers; except that in the latter the pits are more conspicuous, as well as more elaborate, in structure. The large bordered pit, with its valvular torus, is, in fact, typical of coniferous woods. Thick-walled fibres, on the other hand, usually possess vertically compressed, slit-like, bordered pits; while parenchyma cells have simple (*i. e.*, non-bordered pits). These, therefore, are more or less general characters belonging to whole groups of woods. But the position of the pits—whether on the radial or tangential wall—, their relative size, shape and number, and whether in single or multiple vertical rows, are features of some diagnostic value. Thus in larch and spruce the pits of the ray parenchyma cells are typically small and round, or slit-like in shape, whereas in the pines they are relatively large and oval. Again, in some conifers the large bordered pits on the walls of the tracheids are commonly separated by small horizontal folds in the wall surface, known after their discoverer as "bars of Sanio." In cedar the torus—, or central valvular stopper of each bordered pit—is typically scalloped round its edge. In a structurally similar wood *Tsuga heterophylla*, on the contrary, the pit tori have plain edges (the usual type).

Vessel Perforations.—The pitting of the end walls of the conducting vessels is of considerable importance in identification work. It may be of three possible kinds:—

"Multiple" .... *i. e.*,	a number of small, more or less rounded pits.
"Scalariform"	Several transverse slits, giving a ladder-rung appearance to the end wall.
"Simple"	A single large, round or oval, pore, eliminating nearly all the end wall. (Also termed "porous.")

Examples of simple and scalariform vessel perforations have already been mentioned. The type of vessel perforation can sometimes be detected with a good hand-lens under critical illumination, but a moderate power of the compound microscope and thin longitudinal sections are always preferable. (*N.B.* It is an easy matter to confuse the lateral with the oblique end walls of the vessels.)

Marginate Rays.—Seen in longitudinal sections, the storage rays will sometimes be seen to be built up from cells of relatively uniform

size throughout. These are known as "*emarginate*," or "*homogeneous*" rays. In other species, however, the rays are bordered at top and bottom by considerably larger cells. When the latter are sufficiently conspicuous (say about once and a half to twice as high as the remainder) the rays are termed "*marginate*" or "*heterogeneous*."

Such characters are of diagnostic value, and may often just be glimpsed with a hand-lens on a cloven longitudinal-radial section, under intense, critical illumination.

Other features to be looked for in connection with the ray cells are whether they are typically round or oval in cross section (*i. e.*, in a tangential longitudinal section of the wood), and whether they are of distinctly variable size, even though the rays be emarginate.

Reticulate Thickening.—In certain woods irregular "reticulate," or peg-like, thickenings may be observed in the conducting elements; *e. g.*, in the ray tracheids of *Pinus spp.* These deserve note.

Ray Height and Number.—Although both diffuse and uniseriate rays vary in height, either measured linearly or as a count of the number of cells, it is usually possible to determine the *typical* maximum height for a given species—exceptional giant rays excluded. Thus, one may see stated for a given species: "Rays seldom more than 25 cells high."

The width of the rays in cells, where relatively constant, may also sometimes be quoted.

A further feature of less certain specific value (individual variation being frequently excessive) is the average number of rays crossing a given length of the margin of a growth-ring, as seen in transverse section. Or, alternatively, the number of rays per unit area of a tangential longitudinal section may be counted. This is more laborious. (*N.B.* It should be noted that ray number is worthless in respect of woods containing aggregate or compound rays, except in the middle of the wider zones left betwixt the large rays, as the number of small rays is often in inverse ratio to the number of compound rays (*e. g.*, in common oak).

Gum Plugs, Tyloses and Crystal Cells.—*Gummy deposits,* which are common in the vessels and rays of certain woods, especially in tropical species, may be sufficiently specific to be worth recording.

Tyloses consist of small bladders, apparently blown through the pits of the disused vessels by surrounding, living cells. Most commonly found in the heartwood, they rarely occur in the sapwood

also. Tyloses are cellulosic initially, but may later become lignified. When present in quantity, they form a useful specific feature, and may best be seen in longitudinal sections; for in transverse sections they may get broken away during preparation.

A wood such as common oak, which is naturally highly porous, owing to the large size of its conducting vessels, depends for its utility in the manufacture of beer casks upon the presence of innumerable tyloses, more or less completely blocking up the large pores of the heartwood. In false acacia, a well-known ornamental tree, they are still more conspicuous.

Crystal cells are common in many woods, both temperate and tropical, especially amongst the harder genera, generally being found either in the vertical or the ray parenchyma, or both. Even though the crystals themselves (usually composed of calcium oxalate) may become dissolved away during the preparation of thin sections, the cavity, or else the cellulosic framework of the crystal itself, remains distinct, showing its special nature and original crystalline form. Mineral deposits[1] and crystals of various forms will be met with in different species, but cannot be listed here; they should, however, be duly noted, as also cystoliths. Although crystal cells and isolated raphides may be seen by ordinary transmitted light, they can, of course, be better viewed by polarized light against a dark ground.

Pith Flecks, Trabeculae, Stone Cells, etc.—In addition to the foregoing structures and deposits, amorphous aggregates of parenchyma cells—usually extending further longitudinally than transversely—, known as "pith flecks," commonly occur in many woods. Sometimes, as in birch, they come to represent a typical, though sporadic structure, and may be seen on the wood surface as minute rust-coloured patches or streaks. In common oak the writer has observed that pith flecks sometimes appear to exert a co-ordinating influence over the uniseriate rays, causing their fusion into compound organs. Not infrequently they occur chiefly at the margins of the growth-rings; being due, perhaps, to the traumatic action of bark-biting insects—as is also said to be the case with resin ducts in some conifers.

Trabeculae is the name given to a peculiar type of sporadic structure—apparently effecting a transverse strengthening of the cells

[1] *Vide* for example "*Occurrence of Calcium Carbonate Deposits in Wood,*" by S. J. Record, in *Tropical Woods*, 1927. Vol. II, No. 12, pp. 22–26.

concerned—, consisting of dumbell-shaped bars, running in a straight horizontal line across the lumina of a series of contiguous cells. But these structures (which may be encountered in certain conifers, for instance), cannot well be considered of diagnostic importance.

Stone cells (as found in *Piratinera spp.*[1] and the intraxylary phloem of trees such as *Avicennia sp.*), *tannin sacs*, *mucilage pockets*, *latex tubes* (as in the rubber tree and *Apocynaceae* generally), and so on, may also be encountered near the periphery of the stems of woody plants; but these are not often sufficiently important in connection with timber identification to justify detailed description here. Mucilage cells should, however, be noted.

Anomalous Structures.—Besides the anomalous formations of the secondary woody cylinder which are found in the stems of certain lianes, such as *Bauhinia* and *Bignonia spp.*, the wood anatomist will occasionally meet with a wood such as *Strychnos sp.*, for instance, containing patches of what is called *internal, or intraxylary phloem.* In such woods, owing to an atypical behaviour of the active cambium, or meristematic layer located betwixt the bast and the wood, isolated patches of compressed, softer tissue (phloem) are left behind in the midst of the wood. The latter, being at once prominent to the eye beneath a hand-lens, form a useful specific character.

Septate Fibres.—Occasionally the long, thick-walled fibres which form the chief source of mechanical strength in hardwoods, are septate; that is to say, they are crossed at intervals by transverse walls. Such is the case in teak (*Tectona grandis*), for instance. A relatively high power is required for their identification.

Traumatisms and Defects.—All abnormal structures such as those arising from knots, burls and wounds, as well as wavy grain, spiral grain, canted rays, etc. must, of course, be left out of account in the preparation of specific descriptions for the identification of woods.

B. **Physical Methods.**—Thus far we have considered only the more generally useful anatomical features. But, when judging a wood in the field, either without a lens or microscope at all or as an auxiliary to the latter, certain physical features may also be taken into account: These are as follows:

Colour .... Whether almost white, straw-colour, biscuit-colour, light brown, dark brown, chocolate

[1] *Vide* for example "*Note on Sclerosed or Stone-Cell Tyloses,*" Anon., in "*Tropical Woods,*" 1925. Vol. I, No. 4, p. 11.

brown, reddish-brown, grey, black, or some other more striking shade. Note that a descriptive name, such as "dark oak," "canary yellow," or "silver grey" is always desirable in such descriptions.

It should also be stated whether the colour changes, after cutting, with exposure to the air. This occurs in many woods—especially those more highly coloured. *Cæsalpinia sp.* affords an example.

Difference of colour between heart and sapwood also between early ("spring-") and late-formed ("summer-") wood should be noted.

Density. . . . . Woods may be roughly classed as "very light," "light," "medium weight," "heavy," and "very heavy." But care should be taken to ascertain that the samples are first air-dry, as green wood is considerably heavier than seasoned wood. In these terms, balsa would be called "very light," Poplar "light," ash "medium," oak "heavy," and ebony "very heavy."

Strictly speaking, the density of a wood should be given in pounds per cubic foot, air-dry condition.[1]

Hardness. Approximate relative hardness may be estimated by cutting with a sharp penknife. But reliable distinctions can only be drawn from proper mechanical and wood-working tests. (*E. g.*, the impact test, compression parallel to the grain, relative resistance to a standard saw cut transverse to the grain, etc.) For rough purposes, woods may conveniently be classed as "very soft," "soft," "medium hardness" (or "mild"), "hard," and "very "hard." These types, moreover, will generally be found to agree with the corresponding density determinations—the two characters being closely correlated.

Odour. . . . . The smell of freshly cut wood is sometimes sufficiently pronounced to be of specific value. It is, however, extremely difficult to standardise descriptions of such odours, so as to render them significant to other investigators.

Taste. . . . . The taste of a wood, whether "scented," "sweet," "peppery," "resinous," "bitter," "tasteless," etc., may be of occasional use in identification; but the practice is not to be encouraged, as some woods are poisonous.

Fissility. . . . . Some woods cleave well, others not so. Again, those which are fissile are often brittle also. But the character is not a very safe one, as relative humidity and actual physical condition may largely affect the facility with which a wood will cleave. The plane of the cleft, whether radial or tangential, should be stated.

[1] Commonly between 12–15 % of the oven-dry weight in temperate climates.

Lustre	A wood may possess either a generally lustrous or else a *matte* appearance on a fresh-cut surface—especially the radial cut. The relative lustre of the rays in particular, on the radial section (surface view), should also be noted.
Texture	State whether "very fine" (as in box), "fine," "medium," or "coarse" (as in oak), and whether homogeneous or heterogeneous.

In ordinary identification work, and all instances excepting those in which precise quantitative tests upon proper machines are undertaken, the foregoing physical characters may be regarded qualitatively, rather than quantitatively. They are often of crucial value both in field work, where prepared sections and microscopical examination are impossible, and in the final solution of difficult identifications, in which one species or genus cannot surely be distinguished from another, except by a consideration of its macroscopic appearance, density, taste, odour, etc. These characteristics, however, cannot be deduced from charcoals or much rotted specimens.

Specific Gravity.—Although the density of a wood, carefully determined in pounds per cubic foot at a stated % m.c., may afford a fair idea of specific gravity, it is better for diagnostic purposes to calculate the actual specific weight of a sample. The value obtained may then be compared with the accepted range authoritatively quoted for the species or genus. But it must be realised that the latter is usually comparatively wide; wood from different trees, or different parts of the same tree, varying very considerably in specific gravity. In fact, wood from the denser part of a single tree may sometimes be almost twice as heavy as that from more porous portions.

In general, softness and lightness are to be found correlated to a high percentage porosity, and hardness and greater density to a relatively low percentage porosity of the timber; since the specific gravity of dry wood substance is found to be practically constant at 1.56, irrespective of its origin.

But specific gravity also varies very definitely from one species, or genus, to another. Thus extremely porous woods, such as species of balsa, may be lighter than cork, whereas, others—many tropical hardwoods for instance—will not float in water.

Chalk and Rendle[1] define densities of air-dry hardwoods as follows. And the same classification may be used to include softwoods.

[1] "*British Hardwoods. Their Structure and Identification.*" (See Bibliography.)

Light	Less than 35 lbs./cu. ft.
Moderately heavy	35–50 lbs./cu. ft.
Heavy	50–60 lbs./cu. ft.
Very heavy	More than 60 lbs./cu. ft.

Specific gravities of common commercial woods (estimated from the oven-dry weight and "green," or saturated, volume) vary from 0.12 in *Ochroma spp.* (balsa)[1] to 1.37 in *Piratinera guianensis* (Letterwood)[2]—both being hardwoods.

Conifers, which have a consistently high percentage cavity volume (say 50–80% of the whole) mostly fall between 20 and 35 lbs./cb. ft. in density, so that density or specific gravity estimates are not so valuable in identification work for softwoods as for hardwoods, in which the range is much greater.

In estimating specific gravity it is now usual to base one's calculation upon the "oven-dry" weight of a sample (*i. e.*, about 1–3% fibre saturation) and "green" (*i. e.*, 100% f.s., or saturated) volume. The disadvantages of employing air-dry volume is that the moisture content, and hence the volume is not definitely standard. Moreover, oven drying may produce "case hardening" and/or internal collapse of the wood, rendering the apparent volume incorrect. Saturated, or "green," volume is, therefore, usually accepted as standard in specific gravity calculations; while the oven-dry weight is generally near enough to the true desiccated weight for acceptance.

The two points to remember in specific gravity estimations, therefore, are, first, not to oven-dry the wood before its "green" volume has been determined, and second, to state definitely the condition of the wood when the weight and volume were found (*cf. infra*).

A convenient technique for sp. gr. determination, as widely practised, is as follows:—

1. Square the sample parallel to the grain in all three dimensions, being careful not to include an undue proportion of the inner growth-rings (by cutting square, instead of parallel to the grain) when the sample comes near the growth centre of the tree.

If possible, avoid cracked or rotten specimens, as an incorrect volume determination will result. Blocks should be of volume not less than 1 cb. in.

[1] A still lighter wood, sp. gr. 0.044, believed to be *Cavanellesia platanifolia* from Panama, (?), and found on the sea-shore as driftwood, has recently been received at the Imp. Fors. Inst., Oxford from the Forestry Dept. of the Philippines.

[2] Yale collection. According to S. J. Record.

2. Next, boil gently in tap water for several hours, until completely water-logged. If time permits, leave for several days in cold water to swell thoroughly.

3. Employing a rough chemical balance, with large scale pans, determine the volume of the sample by displacement of water as follows.

First, balance a beaker of water, two-thirds full (and large enough to take the wood sample easily) against an equal weight of dry sand (or water) contained in another beaker upon the other pan. Secondly, stick the point of a dissecting needle firmly into one end of the wood sample, and holding the latter under water in the first beaker, clamp the needle. Be careful that the block does not touch the sides of the vessel and is completely immersed.

Finally, re-adjust the balance of the two pans by adding gram and decimal gram weights to the other side.

The total extra weight (in grm.) thus added will then be equivalent to the weight of water displaced by the sample block, which, in turn, may be taken as equal to the volume of the block in c.c.

4. Remove the sample, shake off excess of water, and put to dry in, first, a 60° C.- and then a 100° C.-oven, until the weight, as tested by successive daily weighings, has become constant. *The constant weight may be accepted as the approximate dry weight of wood substance in the sample block.* But if greater accuracy is desired, the sample should, finally, be completely desiccated in a closed container, phorphorus pentoxide being used as absorbant. About 1% m.c. may be atained this way.

The specific gravity of the sample may, of course, now be calculated as usual; being $\left(\frac{\text{the dry weight}}{\text{saturated volume}}\right)$, stated in decimals of unity.

NOTE.—It has been found that *Eucalypts*, such as mountain ash (*E. regnans*), collapse badly on drying, even if the moisture content is high, and the oven- or air-dry methods are therefore inadvisable, since it is unlikely that pieces of wood even from the same stick, will collapse in exactly the same way.

Dadswell (*Commonwealth of Australia, Council for Scientific and Industrial Research. Division of Forest Products*, Tech. Paper No. 2) therefore determined the time of soaking necessary to bring kiln- and air-dry samples to a constant volume (cf. Clarke, *loc. cit.*).

He used samples from the same tree and from different species, the object being to devise a standard general method for identification purposes. Since the loss of weight on soaking (due to solution of extractives) might affect the ultimate oven-dry weight, the latter weight was normally determined by calculation from the mean of these weights for adjacent samples.

Method.—Three adjacent cross-sections, 0.25 by 0.5 inch thick, are cut at least 2 inches from one end of the sample, and held together, and trimmed to about 2.5 × 1 inch, so that only sound heartwood is obtained. The 3 samples are scraped free from splinters and sawdust and placed in an air-tight container, the 2 outer pieces (M) then being weighed together (a grms.), and again after drying at 105° till constant in weight (b). The middle piece (D) is then weighed (c), immersed in water till constant in volume (about 6 days), wiped dry, and the volume (V) determined by displacement in the usual way *i. e.*, from the buoyant force exerted by the sample on total immersion in water. Then oven-dry weight of $D(= W) = bc/a$, and specific gravity $= 62.5W/V$ in pounds per cubic foot. The oven-dry weight of D when re-dried was also recorded, this value for 18 different species being 0.03 to 0.75 grm. less than that calculated from the two adjacent samples (M).

Results.—It was found that soaking for 5–6 days was required to obtain a constant volume and that soaking, even for *E. regnans* dried at 105°, brought collapsed samples back approximately to their "green" dimensions.[1] The objections raised against other methods are therefore avoided. The data showed that slight collapse can occur even with very high moisture contents, so that it is advisable to soak even green samples (100% of water) to ensure the maximum volume being attained. Comparison with other methods showed that the new procedure gave more uniform results. In particular, for karri (5 samples) and jarrah (23 samples) the oven-dry weight/volume method gave values ranging from 46.8 to 56.8 and 43.8 to 58.5 lbs./cu. ft., respectively, whilst the ranges for the new method were 37.9 to 46.1 and 38.9 to 45.8, respectively. The greater variation with the old method is due to the effect on the oven-dry volume of uneven shrinkage prior to and during drying.

In cases where the sample available is too small to permit the above method of sampling the oven-dry weight should be deter-

[1] The writer has found the same thing with collapsed samples of S. African and American swamp oak.

mined first, and the same piece used for soaking. The reverse procedure is objectionable on account of loss in weight due to material soluble in water, and the procedure suggested is not suitable for general use since some woods may split during drying, and it is not yet established that all oven-dry woods will regain their "green" volume on soaking.

The Polarising Microscope.—Broadly speaking, the polarising microscope will be found more useful as an aid to special researches into the structure of the cell wall, in the examination of fibres, trabeculae, states of mechanical strain and failure, etc., than for general identification and everyday purposes. The polariser will, however, sometimes be found helpful in detecting crystal cells, as already noted, as well as for giving a clearer picture of certain tissues —thick-walled fibre groups, for example.

In woods, as a rule, the lignified portions of the cell wall are optically active beneath the polariser, and exhibit right extinction; whereas the softer, cellulosic parts appear inactive.

Micro-Projection and Photomicrography.—For rapid survey and examination of thin sections, in areas up to 0.5 inch sq., a small microprojector, such as those supplied by Messrs Leitz of Wetzlar and various other manufacturers, is invaluable. This type of instrument can also be used for low power photography;—the use of bromide paper and suitable colour filters (a tri-colour green is good with either safranin-haematoxylin or violet-orange preparations) enabling very fair, large-field negative prints to be made from stained sections in less than a minute. Magnifications from × 10 to × 60, or so can be obtained this way. For higher powers the use of a good compound microscope and an arc lamp, or pointolight, with panchromatic plates is recommended.

For finer low-power photographs on negative plates, or on bromide paper, special large field, low-power objectives may be employed. (*E. g.*, the "Microsummar" lenses supplied by Messrs Leitz.)

But a standard micro-projection apparatus, without any special photographic lenses or optical bench, will be found very satisfactory for general work.

Next to Ilford "Special Rapid Panchromatic," Imperial "Eclipse Ortho." plates, though fast, are to be highly recommended; they are also cheaper. But for best results panchromatic plates with

suitable colour filters cannot be surpassed. Backed plates are preferable for fine work, especially where a certain amount of glare (as in photography of charcoal sections) is unavoidable. But for the latter purpose—where the tissues are black and opaque—a slow, fine-grained plate may sometimes be used to advantage; Ilford "Process" plates, for instance. Wellington "Anti-screen" plates are also much used with a pale yellow-green filter for general photomicrographic purposes.

Micro-projection and low-power photomicrography are of great assistance to the wood anatomist, not only for purposes of public demonstration or record, but also as enabling a more general and leisurely survey of the structures under examination than can conveniently be made with a compound microscope.

C. **Chemical Methods.**—Macroscopic and microscopic methods of identification are frequently not applicable to closely related species of the same genus, *e. g.*, the Australian hardwoods of the genus *Eucalyptus*, over 300 species of which are known to exist, many of these being cut for marketing.

Chemical methods have therefore been devised, though here again, a limit to their value is set by possible variations in composition within the same species, and by the difficulty of obtaining authentic samples for formulation of the test and for control experiments.

The chemical methods fall into two main classes: (1) Quantitative analysis of the composition; (2) simple qualitative tests based on characteristic differences in composition. The former may be regarded as supplementary to the latter in cases of doubt.

Kanehira ("Identification of Formosan Woods," *Bureau of Productive Industries, Govt. of Formosa*, 1921) developed an "identification key" based on colour, flavone content, and the fluorescence after extraction of the wood for 14 days with old water or ammonia. In the flavone test 10 c.c. of an extract in alcohol are acidified with concentrated hydrochloric acid and reduced by the action of magnesium powder and a small drop of mercury. The colour develops on standing, but such methods do no more than allow a wood to be placed in one of 3 categories.

[1] The writer wishes to acknowledge his indebtedness to Dr. Julius Grant for his valuable assistance in summarising previous work on the Chemical Identification of Woods.

Welch (*J. and Proc. Royal Soc. N.S.W.*, 1922, **56**, 241) noted the colours and precipitates produced by the addition of reagents such as ferric chloride, ferrous sulphate, lime water and sodium hydroxide solutions to extracts of wood-shavings from ironbark *Eucalypts* in water or alcohol. Only comparative results for the different species are given, and it is difficult, or impossible, to use these for identification purposes without thorough systematisation of the method of procedure and classification of the results.

Distinction of Genera and of Heart- and Sap-woods by Chemical Analysis.—The work of Ritter and Fleck (*J. Ind. Eng. Chem.*, 1923, **15**, 1055; 1926, **18**, 576) has made possible the classification of a number of American hard and soft woods, and has also enabled heart-woods to be distinguished from sap-woods.

Comparative analyses were carried out by the authors' methods (*ibid.*, 1922, **14**, 1050) on white ash, yellow poplar, pignut hickory, yellow birch, white oak, bald cypress, white pine, and yellow, white and incense cedars, and the following generalisations were drawn from the results:—

Ash Content and Methoxy Groups.—There was no general relationship between the results. *Extractives.*—(*a*) Softwoods: In general, for ether, cold or hot water or 1% sodium hydroxide solution the sap-wood gave lower values than the heart-wood. (*b*) Hardwoods: (i) the same rule applied to yellow birch, white oak and yellow poplar, and (ii) the reverse to white ash and pignut hickory. *Acetic Acid* (by hydrolysis).—The sap-wood values were greater than those for heart-wood from both hard and soft woods. *Pentosans in both Wood and Cellulose.*—Sap-woods gave slightly higher values in both cases. *Methyl-pentosans in Wood and Cellulose.*—In both cases, for the same class of wood, there was a tendency for the heart-woods to give slightly higher values. *Cellulose contents* were grouped similarly, but in the reverse sense, to the extractives, *e. g.*, for the softwoods the sap-wood values were greater than the heart-wood values. Two exceptions (yellow poplar) were attributed to the condition of the sample, since owing to decay of the sap-wood the proportion of extractives had been increased. Yellow poplar, therefore, really belongs to the first group of hardwoods (*vide infra*) the high cellulose figure for the heart-wood compared with the sap-wood being explained probably by reduction of cellulose in the latter case by decay.

Lignin.—Softwoods: sap- greater than heart-wood, except for white cedar. Hardwoods: heart- greater than sap-wood, except for yellow poplar. *Ratio of OMe/Lignin.*—The ratio for hardwoods was approximately 50% greater than that for softwoods. For bald cypress it is below 13%, and this wood must, therefore, be distinguished, as far as chemical composition is concerned, from red wood and live-oak, for which the methoxy value (*loc. cit.*) is 17.5%. α-, β- and γ-*Cellulose* contents showed no definite groupings.

Subsequent experiments (*loc. cit.*, 1926) on the same lines confirmed the above methods of grouping according to the yields of extractives on the one hand, and the cellulose and lignin values on the other. White spruce, however, was an exception to the general rule that high extractives correspond with low lignin and cellulose contents (and *vice versa*), since the two latter values for the heart- and sap-woods were approximately equal in this case.

The hardwoods examined may therefore be classified as follows:—

A. (higher sap-wood extractives):
White ash, pignut hickory, red alder, red mulberry, sugar maple and catalpa.

B. (higher heart-wood extractives): Yellow poplar, yellow birch, white oak, locust and eucalyptus.

Hemlock, redwood and red oak, and the bald cypress, white pine and yellow, white and incense cedars previously examined (*loc. cit.*, 1923) all had higher cellulose contents.

Distinction of Spring-wood from Summer-wood.—Samples of spring- and summer-woods, taken from different portions of the annual growth rings differ in appearance, cell structure, density and cell-wall thickness. Chemical analyses were carried out on Douglas fir, loblolly and western white pines, catalpa, red alder, white ash (sp. gr. 0.68, 0.71 and 0.81) by Ritter and Fleck (*ibid.*, 1926, **18**, 608) with the object of providing means of identification.

Bands were cut from cross-sections, 3 mm. thick, though difficulty was experienced in obtaining representative samples for consecutive years on account of variations in the width of the rings from season to season. The spring and summer woods were separated by means of a chisel, then ground to 80–100 mesh, and were analysed by the authors' methods (*loc. cit.*, 1922).

In general, the lignin content was found to be uniformly greater and the cellulose less in the spring-wood, except for the heart-woods

of loblolly pine and red alder, where the cellulose also was greatest for the spring-wood. These general conclusions may be explained (cf. Ritter, *ibid.*, 1925, **17**, 1194) by the fact that the lignin occurs partly in the cell-wall (accompanied by cellulose), and partly in the middle lamella, of which it is the principal constituent. If, therefore, the ratio of lignin to cellulose in the cell wall (*i. e.*, excluding the lamella) is the same for the spring-wood as for the summer-wood, then the lignin in the lamella (which constitutes a large proportion of the total wood) would account for the greater proportion of lignin in the spring-wood.

Apart from this, few uniform differences in chemical composition were observed, though there was a tendency towards higher percentages of pentosans and extractives in the spring-wood as compared with the summer-wood, and of pentosans in the cellulose from the spring-wood.

Distinction of Closely Related Species of the Same Genus (Eucalyptus).—Dadswell (*Commonwealth of Australia, Council for Scientific and Industrial Research, Division of Forest Products, Tech. Paper*, No. 1, 1931) has pointed out that the methods of chemical analysis described by Ritter and Fleck (*J. Ind. Eng. Chem.*, 1922, **14**, 1050) will not necessarily reveal differences between closely related species of the same genus, since they depend on the determination of extractives which may differ in nature, though not in amount. Moreover, as Ritter and Fleck have shown (*loc. cit.*), the extractives may also differ according to whether they are derived from the heart- or sap-wood. These considerations apply particularly to the genus *Eucalyptus*, some species of which are wood-structurally identical, and cannot be distinguished by microscopical methods.

As a first attempt to find distinguishing chemical tests, the samples (80–100 mesh) were extracted with hot water or alcohol according to the standard procedure, the liquids diluted so as to be equivalent to 1 grm. of oven-dry wood in about 150 and 30 c.c., respectively, and tested on a white spotting-tile with equal volumes of 1% solutions of ferric alum and chloride, ferrous sulphate, lime water, potassium hydroxide and lead acetate. No reliable results were obtained with karri, jarrah or the ironbark woods, the colours being similar throughout and due probably to the same tannins in each

of the species, any small differences being explained by variations in the solubilities of the extractives.

Methods suitable for use with certain closely-related species were, therefore, developed as follows:—

Karri and Jarrah.—(1) *Cellulose Determinations.*—An 80–100 mesh sample (*i. e.*, passing an 80-mesh but not a 100-mesh sieve) was analysed by the standard procedure of the U. S. Forest Products Laboratory (Schorger, "*Chemistry of Cellulose and Wood*," 1926; Hawley and Wise, "*The Chemistry of Wood*," 1926). For 34 authentic samples of Western Australian jarrah and 17 of karri (all indigenous) the percentage of cellulose on an oven-dry basis was 38.4–51.6 (mean 44.5), and 53.0–62.7 (mean 59.0). This test, therefore, enables the two species to be distinguished, though it is not easy to carry out.

(2) *The Burning Splinter Test* (Swain, *J. of Forestry*, 1924, **24**, 754 *et sq.*) has been claimed (Anon, *Aust. For. Jour.*, 1926, **9**, 17) as a means of distinguishing between jarrah and karri, though Campion (*ibid.*, 1927, 10, 154) criticised it adversely on the grounds that the results obtained depend on the experimental conditions (*e. g.*, size and water-content of the splinter). Dadswell (*loc. cit.*), however, found that if splinters the size of half a match were taken from the sound untreated heart-wood and burnt, those from over 150 samples of karri from different sources burned well, glowed for a long time, and left a white ash, whilst jarrah (200) burned poorly without glowing and left little or no ash which, when it was obtained, was black. He failed to confirm Campion's criticisms, but found that the test might be upset if sound heart-wood is not used, or if the wood is weathered or treated with a preservative.

(3) *Alkalinity of the Ash.*—The percentage of ash in 5 grms. of sawdust from the weathered wood was determined by ignition in a platinum dish; a known (excess) volume of 0.1 *N* hydrochloric acid was then added (5–10 c.c.), and the mixture was warmed. The alkalinity was determined by titration of the excess with 0.1 *N* sodium hydroxide solution, with phenolphthalein as indicator.

The results, calculated on the oven-dry weight of the original wood, and the minimum alkalinity value of karri, were shown to be more than twice the maximum for jarrah. Here again, however, sound heart-wood should be used, since weathered jarrah may give an

kalinity within the karri range. It seems that weathering proices changes in the chemical composition which are reflected in the h.[1]

Tallowwood, Blackbutt and White Mahogany.—(1) *Alkalinity the Ash.*—The species examined were from the coastal areas of N. Wales and S. Queensland. Except in one abnormal case, where e alkalinity was 0.06 c.c., tallowwood ash gave higher values than ose from the two other species.

(2) *The Burning Splinter Test* corroborated the results of (1), *i. e.*, lowwood gave a grey-white ash, and the others a carbon tip.

(3) *Dilution Test.*—About 2 grms. of sawdust were heated with c.c. of alcohol for 2 minutes, the cool extract was filtered, and a rtion was diluted with an equal volume of water. Tallowwood oduced *immediately a definite white unfilterable turbidity;* blacktt gave a clear solution (and a precipitate after some time); iite mahogany produced an immediate turbidity, which, however, s filterable, giving a clear solution. This test, in conjunction th (1) and (2), enables the 3 species to be distinguished; it is sed *on the waxy nature of the ether-soluble extract of tallowwood,* distinct from those of the others. Solutions of these extracts in ohol also give the test.

Ironbark Group.—(1) *Alkalinity.*—In general, a high alkalinity licates *E. paniculata* or *crebra*, and a low value one of the other ecies. The cellulose content of the former (49.0–56.0%) is unimly higher than that of *E. crebra* (35.4–46.3).

(2) *Dilution Test.*—*E. sideroxylon* gave an immediate precipitate h *a slight fluorescence; E. siderophloia, propinqua* and *punctata* ve clear solutions. No means are at present available to disguish *E. siderophloia* from *E. propinqua* or *crebra.*

Red Box (*E. Polyanthemos*) **and Red Gum** (*E. Rostrata*).—The wdust was extracted first with hot water and then with alcohol, c.c. of 1% sodium hydroxide solution was added to about 5 c.c. the alcohol extract, and the mixture was placed in a water-bath. yellow precipitate, leaving a yellow-green supernatant layer with slight fluorescence, and a chocolate precipitate were obtained, pectively, with 7 samples of each species, and no exceptions were

[1] Similarly, the present writer has observed that the percentage of tannin, the relative roscopicity (and, hence, the percentage swelling and shrinkage with changes of atmosric humidity), the percentage of mineral ash, and the specific gravity may be consider- modified in common oak wood by fungal attack. The general microscopic structure, ever, may remain unaltered, and the wood still look relatively sound.

observed. It was proposed to extend these studies to other botanically similar groups of woods.

Determination of Lignin.—In the method suggested by Mahood and Gable (*J. Ind. Eng. Chem.*, 1922, **14**, 251) and adopted by the United States Forest Products Laboratory, 2 grms. of the air-dry sawdust, ground to pass an 80 to 100-mesh sieve, are extracted for 4 hours with the mixture of alcohol and benzene (1:2) having a minimum b. pt. This should remove gums, kinos, resins, etc., and is followed by digestion of the dried residue with cold 72% sulphuric acid for 16 hours. The acid is finally diluted to 3%, and the residue, after further digestion for 2 hours by boiling under a reflux condenser, is filtered off on a tared alundum crucible, washed neutral with hot water, dried at 105° C. and weighed as lignin.

Ritter and Fleck (*J. Ind. Eng. Chem.*, 1922, **14**, 1050; 1923, **15**, 1055; 1926, 18, 576, 608) obtained in this way of values about 25% (on the oven-dry sample) for numerous North American hardwoods. Cohen and Dadswell (Commonwealth of Australia Council for Sci. and Indust. Res. "*Chemistry of Australian Timbers*," *Technical Paper*, No. 3, 1931), however, in applying the method to Australian hardwoods of the genus *Eucalyptus*, obtained abnormally high and varying values in most cases, *e. g.*, *E. marginata* 38.9–54.5%, *E. sideroxylon* 28.0–40.5%, *E. propinqua* 34.2–38.8% etc. Microscopical examination of thin sections of the wood showed large quantities of dark-coloured substances in the wood parenchyma, ray cells, vessels and even in the lumina of the wood fibres, and it was concluded that the preliminary extraction was ineffective in such cases, the final lignin being contaminated with these substances. The solubility of the contaminants was found to vary considerably for one species, *e. g.*, from 8 to 29.5% for red ironbarks.

Other workers have also condemned benzene, with or without alcohol, as universal preliminary extracting agents, though Dore (*J. Ind. Eng. Chem.*, 1920, **12**, 472, 476) found them suitable for conifers. Some use dilute alkali alone or in conjunction with one or both of these solvents; *e. g.*, Friedrich and his co-workers (*Monatsh.*, 1925, **46**, 31; 1926, **46**, 597) and Horn (*Cellulosechemie*, 1930, **11**, 151) who extract resins with benzene-alcohol and gums with 5% sodium hydroxide solution. Bechmann, Liesche and Lehmann (*Biochem. Zeit.*, 1923, **139**, 491) used alkali in conjunction with ether, but obtained abnormally low results (about 3%), and Schorger

("*Chemistry of Cellulose and Wood*," 1926) suggested dilute ammonia. Opinions in the past have differed as to the advisability of alkali as an extracting agent, since, as Pringsheim and Magnus point out (*Z. physiol. Chem.*, 1919, **105**, 179), the presence of acetates after such treatment was taken with some assurance as an index of the presence of acetyl groups in lignin (see also Dore, *loc. cit*). The possibility of removal of lignin by the alkali was also suggested by Schorger, though disproved by Ritter (*J. Ind. Eng. Chem.*, 1925, **17**, 1195) and, to some extent, by Hawley and Campbell (*ibid.*, 1927, **19**, 742) and Ross and Hill (*Pulp and Paper Mag.*, Canada, 1929, **27**, 15, 541; 1930, **29**, 13, 453) who showed that with sitka spruce, balsam fir, poplar, chestnut and cherry the loss on extraction with a 1% solution of sodium hydroxide at 100° was practically constant after 6 hours, and that even after 24 hours there was a definite ratio of cellulose to lignin in the residue. These workers, and Campbell and Booth (*Biochem. J.*, 1930, **24**, 642) who worked on the heart- and sap-woods of English oaks, found lignin values lower than those given by the standard method.

In view of the inconclusive results, due probably to the use of woods of different origins, Cohen and Dadswell (*loc. cit.*) examined exhaustively the effects of various solvents on the *Eucalypts* under varying conditions of concentration and time. In the case of neutral solvents (benzene containing 33 and 50% of alcohol, ethyl and methyl alcohols, ether, carbon tetrachloride, acetone, hot and cold water, sodium and ammonium acetates and ammonium oxalate) the wood was exhausted in a Soxhlet apparatus or in open beakers, and the residue was examined microscopically for extraneous matter, and chemically for the apparent lignin content. Although in some cases 12–15% of the wood powder was removed from red- and narrow-leaved iron-bark, jarrah, red mahogany and Canadian hemlock, the extraneous matter was not completely dissolved. The apparent lignin content of the hemlock was 29.2–29.8 whether benzene-alcohol (2:1) or alcohol, alone or after hot water, was used, and this, in spite of the fact that the amounts extracted by these solvents showed considerable variation.

On the whole, the results were negative, and the effects of alkaline solutions (1–35% ammonia, 2% sodium sulphite and bisulphite, 2–5% sodium carbonate, 0.05–1.25 *N* sodium hydroxide, 5% sodium biborate and 1–2% lime water) were, therefore, tested at 20–100°

for 1–72 hours. Qualitative experiments showed that sodium hydroxide alone could be used for the complete removal of extraneous matter, though extraction with a 1.25 *N* solution in the cold for 72 hours according to Friedrich (*loc. cit.*) was found to be unsuitable for routine analyses. Quantitative experiments with 0.05 *N*–0.125 *N* solutions were therefore made, the sample (3 grms.) being heated on the water-bath with 100 c.c. of solution (150 c.c. for jarrah) in a 300-c.c. conical flask fitted with an air-condenser for 20–320 minutes. The residue was filtered on a tared alundum crucible, washed with hot water, 10% acetic acid, and again with hot water till free from acid, and the residue dried at 105° and weighed. The lignin in the residue was determined by the acid method (p. 483).

The results were followed microscopically and also plotted as curves showing the change with time of (1) extracted matter, (2) lignin, for the strengths of alkali used and for the standard benzene-alcohol solvent. Both methods confirmed the incomplete extraction by the latter solvent and showed that optimum conditions were obtained with 0.125 *N* sodium hydroxide solution acting for 80 minutes at 98–100°. Less drastic treatment may fail to remove all the extraneous matter in a given time, whilst stronger solutions or longer periods are unnecessary, since, in general, they do not give a different lignin figure than the conditions suggested, and may introduce the possibility of attack of the wood-structure.

Sampling.—Cohen and Dadswell (*loc. cit.*) also found that the standard method of sampling was not applicable to the *Eucalypts*, since the extraneous matter already mentioned is very brittle, and forms in the mill a fine powder which tends to pass preferentially through the 100-mesh sieve, so that an unduly large proportion is obtained in the rejected portion. This conclusion was supported by determinations of lignin and microscopical examination of the portions of a sample of iron-bark, (1) 80–100 mesh, (2) passing the 100-mesh sieve (*i. e.*, rejected), and (3) after (1) had been ground further so as to pass the 100-mesh sieve completely. It is suggested that this error may apply even to soft woods, and the procedure suggested is that an entire block, cut from the tree and sawn into strips, should be ground in a laboratory impact-mill until as much as possible passes through the 100-mesh sieve. If 200 grms. are used, the error caused by rejecting the small amount of ungrindable material is negligible. The mixed sample may then be reduced by quartering.

Results.—These refer to Cohen and Dadswell's conditions, and give, in order, the percentages of extracted matter and lignin, based on the oven-dry weight of the original sample.

Canadian hemlock, 13.7, 26.4; Canadian spruce 13.0, 24.8; jarrah (*E. marginata*), 37.2, 21.7; red ironbark (*E. sideroxylon*), 36.1, 20.5; mountain ash (*E. regnans*), 18.0, 21.4. Analogous figures for the benzene-alcohol (2:1) mixture are 2.2, 30.1; 0.9, 28.4; 0.9, 50.6; 5.6, 42.3; and 2.3, 28.1, respectively. The lignin in hemlock and spruce is more resistant to the action of the alkali than that in the other woods, though in no instance is the accuracy of the method appreciably affected.

Distribution of Chemical Compounds.—The chemical aspect of the subject, as a whole, may best be surveyed by summarising the distribution of the various classes of chemical compounds in the woody parts of trees.

The principal classes of chemical substances occurring in plants are *carbohydrates*, *vegetable acids*, *fats*, *aromatic compounds*, *proteins*, and *plant bases*. Of these, a number occur in woody tissues, and are thus of interest to the wood technologist, as well as of occasional utility in identification work.

The Carbohydrates.—This is the most important group, including, as it does, those complex secondary products of metabolism, the *sugars* and their condensation compounds, the *celluloses*, *lignins*, *starches*, *dextrins*, *gums* and *mucilages*.

(a) **Cellulose and Lignin.**—Celluloses, ligno-celluloses and cuto-celluloses form the basic cellular framework of every woody plant. They are, therefore, of no specific or diagnostic value, except quantitatively, as has been shown above (pp. 478–486).

(b) **Starch.**—The form and abundance of starch granules in the storage parenchyma, rays, and especially in the roots of a tree, may sometimes be worth consideration. The relative amount of starch reserves, on the other hand, being variable from part to part, from tree to tree, and from season to season, is not really a reliable characteristic. Only where the quantity or form (*e. g.*, dumbell-shaped grains) is sufficiently remarkable and consistent from specimen to specimen, should starch be utilised as a specific feature of the wood.

Starch may, of course, be detected in the usual manner, by means of an alcoholic tincture of iodine.

(c) **Gums.**—These are often of specific or generic character. For instance, gum arabic (arabin) occurs as an exudation from the branches of *Acacia senegal*, and other members of the same genus. Gum tragacanth is obtained from the stems of several tragacanth shrubs—chiefly *Astragalus gummifer*—, members of the order *leguminosae*. Cherry gum (cerasin) exudes from the bark of the cherry (*Prunus cerasus*), the bird cherry (*P. padus*), the plum (*P. communis*), the almond (*P. amygdalus*), and other members of the *Rosaceae*. On hydrolysis it is stated to yield almost entirely arabinose (see also Vol. I, p. 556 *et seq.*

(d) **Sugars.**—Plant sugars, though present mainly in the fruits, also appear in other parts of the root and shoot system, as transition products to starch; also as pentosans, (*e. g.*, araban) in various gums. One of the most widely distributed is *glucose*. Others are *xylose*, which is common in woody tissues as the pentosan, xylan; *rhamnose; galactose*, fairly widely distributed as galactans in various gums, mucilages, etc.; *mannose*, as condensation products, mannans, but seldom free; *laevulose* (or fructose), present in various parts of a great many plants; *maltose*, formed by the hydrolysis of starch, and therefore widely distributed; *sucrose* (cane sugar) also widely distributed in plants, inclusive of the stems.

In addition to these mono- and disaccharides, there are also several tri- and tetrasaccharides, such as *raffinose*, *rhamninose*, *gentianose*, *stachyose*, and *melicitose;* which last is of importance, being found exuding, as a manna, from the twigs of larch (*Larix europaea*) and Douglas fir (*Pseudotsuga taxifolia*) (See also Vol. I, p. 358).

(e) **Pentosans.**—*Araban*, already mentioned as a condensation product of arabinose, is present in various gums. Another pentosan, *xylan*, occurring in lignified cell walls of many trees, excepting conifers, represents the chief constituent of "wood gum"; on hydrolysis it yields xylose.

(f) **Mannans.**—These condensation products of mannose occur in many seeds, as well as in certain bulbs and tubers, as constituents of mucilages; also in the wood and leaves of certain trees. They may, therefore, occasionally be of specific value. Most mannans are readily hydrolysed by dilute hydrochloric and sulphuric acids.

(g) **Galactans.**—Galactans are condensation products of galactose. They occur principally as constituents of certain gums and

mucilages, and in the reserve tissues of seeds, but need not be considered in detail here.

(h) **Pectins.**—Pectins are found to occur chiefly in the juices of succulent fruits, stems and roots. But a pectic substance, known as "pectinogen," probably also occurs in many cell walls in combination with cellulose. It will not, however, possess specific value in wood identification except, perhaps, quantitatively. (*Cf.* pp. 509 *et seq.*)

Fats and Allied Substances.—In this group fall the fixed oils, waxes, lecithins and sterols. Oil cells (together with mucilage cells) are to be found in various woods such as those of the *Lauraceae*, *e. g.*, in *Cinnamomum spp.* Thus Janssonius[1] states that oil cells were found in almost every member of this family so far examined by him.

Oils turn black with osmic acid: alkannin, sharlach red, or Sudan III may also be employed as alternative tests, each of these staining the oil, or fat, a typical pink colour.

Aromatic Compounds.—Of the aromatic compounds, those which may be mentioned are as follows:—

(a) **Phenols.**—*Phloroglucinol* (which, followed by hydrochloric acid, forms so valuable a test for lignin), is found widely distributed in the combined state in various complex plant substances, but also uncombined. If present in wood, it may, therefore, be detected by the application of hydrochloric acid—productive of a typical, fine red coloration. (See also Vol. V, p. 17.)

(b) **Inositol.**—This substance is soluble in water, but crystallises out on addition of strong alcohol. It is widely distributed in plants, especially in young leaves and growing shoots, where it might possibly be encountered by the analyst. (*E. g.*, in walnut?)

(c) **Aromatic Acids, Aldehydes and Alcohols.**—*Salicylic alcohol* occurs as the glucoside, salicin, in the bark of certain species of willow, where it might be of diagnostic value.

Gallic acid, which forms a constituent of many tannins, occurs in the bark of some trees, common oak, for instance. It is fairly soluble in hot water, and in alkaline solution rapidly absorbs oxygen from the atmosphere, turning brown. It is a product of the hydrolysis of gallotannin (see Vol. V, p. 18).

(d) **Tannins.**—Tannins, which are widely distributed throughout many genera and species of plants, are, nevertheless, sometimes

1 "*Mucilage Cells and Oil Cells in the Woods of the Lauraceae*," H. H. Janssonius; in Tropical Woods, Vol. I, No. 6, pp. 3–4. 1926.

absent. Further systematic investigation, however, is still required to render them of use in identification work.

They generally occur in solution in the cells of various parts of the plant, including the woody parts of root and shoot. In some instances special tannin sacs are provided in the softer tissues.

Gallotannin tends to collect in dead tissues, such as the bark and the heartwood of trees; the bark especially is so richly stocked as to be of commercial value in the leather industry. Thus the following species are noteworthy:—*Caesalpinia spp.*, *Castanea sativa* (sweet chestnut), *Eucalyptus spp.*, *Quercus spp.* (oak), *Rhizophora spp.* (mangrove), and *Rhus spp.* (sumac). Oak galls are also rich in tannin, as is well known (see Vol. V, p. 50).

Tannins possess an astringent, bitter taste, give blue or green colours with iron salts, are precipitated by potassium dichromate, and will precipitate gelatin from solution. They are non-crystalline, and exist in solution in the colloidal state.

Not all tannins give the same reactions; they can, therefore, be classified in groups—a fact of probable significance in identification work on timbers. There are the *pyrogallol tannins*, which give a dark-blue coloration with ferric chloride and no precipitate with bromine water, and the *catechol tannins* which give a greenish-black colour with iron salts and a precipitate with bromine water. Tannins of the first type may be extracted from the bark of oak, sweet chestnut and sumac; whereas the bark of horse chestnut (*Aesculus hippocastanum*), of walnut (*Juglans regia*) and of larch (*Larix europaea*) produce catechol tannins (see Vol. IV, p. 21).

(e) **Essential Oils and Resins.**—The mixtures of organic volatile products known as "essential oils"—including *terpenes*, *alcohols* (derived from the terpenes), *esters* of these alcohols and *phenols*—should, perhaps, be reckoned as the most important chemical group, considered from the identification standpoint. Thus "oil of turpentine," containing *pinene*, is obtained from *Pinus*, *Larix* and *Abies* spp.; whilst "camphor oil" is derived from the solid terpene, *camphene*, from the camphor tree (*Cinnamomum camphora*). Since oily woods also commonly possess a typical odour, it will usually be simpler to employ the latter as an aid to identification rather than to attempt actual extraction of the oil itself. (See also Vol. IV, pp. 1–217.)[1]

[1] *Note.*—It may be mentioned here, as an example of the value of anatomical knowledge, that the supply of sandalwood being limited, and the demand on the increase, several sub-

Cedrol a crystalline derivative of cedar oil can be isolated from the cedar wood of old black-lead pencils (Mitchell, *J. Soc. Chem. Ind.*, 1919, **38**, 138T).

Resins are oxidation products of the terpenes. They may be classed as hard resins and balsams; *e. g.*, copal and dammar on the one hand, Canada balsam and turpentine on the other. (See also Vol. IV, pp. 219–359.)

Other "essential oils," such as "clove oil" and "lemon oil," which are obtained from the non-woody parts of certain plants need not be considered here.

(f) **Flavone and Flavonol Pigments.**—These have been obtained chiefly from flowers; though more rarely they sometimes also occur in the harder portions of plants; as, for instance, *quercetin* a very widely distributed yellow dye, which is present in the bark of certain oak species.

(g) **Anthocyan Pigments.**—These constitute almost all the blue, purple and red colours of plants, and may be found in the cell sap of stems, as well as in the softer parts, such as flowers. As glucosides, they are termed anthocyanins.

With acids the anthocyan pigments give a red colour; with alkalies, usually blue or violet, if pure, or green when mixed with flavones or flavenols. In an aqueous solution of alcohol the anthocyan pigments lose colour by isomerisation. With lead acetate they give insoluble precipitates—blue, if pure, otherwise green. Finally, nascent hydrogen has the property of temporarily removing the colour of these compounds, which reappears on exposure to air.

Fortunately, the reactions of the anthocyan pigments show some specific differences from plant to plant, though details cannot be given here. They may, therefore, sometimes be of value in wood identification.

Logwood dye (haematin), obtained from *Haematoxylon sp.*, which is so extensively used in biological work, may be cited as an example of a pigment, giving definite reactions, extracted from wood.

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stitutes have been marketed—as also in the notorious case of mahogany—of which the better known are as follows:—

Fusanus spicatus............................Australian sandalwood.
Esenbeckia alata............................West Indian sandalwood.
Amyris balsamifera..........................Venezuelan sandalwood.
Osyris tenuifolia............................East African sandalwood.
Mansonia gagei..............................Burmese sandalwood.

In fact, the last—a member of the *Sterculiaceae*—is frequently confused in commerce with the true sandalwood of India. This and all other substitutes may, however, be distinguished from wood of *Santalum album* itself both by their structure and their smell.[1]

[1] *Vide* "*Sandalwood and Its Indian Substitutes*," by K. A. Chowdhury. Indian Forester, Sept. 1931, Vol. 57 (9), pp. 431–433; J. C. Maby (*Anaylst*, 1932, 57, 515).

(h) **Oxidases.**—These plant enzymes appear to be present in only about 63% of the higher plants. Usually the genera of a given order are all either "oxidase plants" or "peroxidase plants"—*i. e.*, lacking the oxygenase and catechol elements. The large and important order of the *Leguminosæ*, which entails so many timber trees of the world, however, contains members of both types.

After death, injury or treatment with chloroform vapour, etc., *oxidase tissues* turn brown, or reddish-brown, in air. *Peroxidase tissues*, on the contrary, show no such effect.

A number of commercial and tropical woods exhibit this phenomenon; *Chlorophora excelsa* and *peltogyne sp.* ("Purpleheart"), for instance. Thus, too, the latex of *Rhus vernicifera*, the lacquer tree, darkens on exposure.

As test for peroxidases, guaiacum gum (obtained from two valuable W. Indian trees, *Guaiacum officinale* and *G. sanctum*), used in 10% alcoholic solution, gives a typical blue coloration when oxidised. The solution should be used fresh, as guaiacum tends to form peroxidases itself on exposure to air.

The enzyme found in the lacquer tree, noted above, is said to be *tyrosinase*. Other specific enzymes need not be mentioned here. (See also Vol. VIII, pp. 151–192.)

Glucosides.—Passing over the vegetable acids and the proteins and amino acids, as have little bearing on the present purpose, next we come to the glucosides, which may be regarded as ester-like compounds of carbohydrates with aromatic substances.

Briefly, one may mention that, on boiling with dilute acids, or as the result of the action of enzymes, glucosides split up into a glucose (or other sugar) *plus* another organic constituent. For details of the chemistry of glucosides, a standard biochemical work should be referred to (see Bibliography, and Vol. VIII, pp. 1–78).

The following glucosides occurring in woody genera may be cited by way of example.

Glucoside	Order or genus
Aesculin	*Aesculus sp.*
Amygdalin	*Prunus sp., Pyrus sp.*
Coniferin	*Coniferae.*
Cyanin	*Rosa sp.*
Fraxin	*Fraxinus sp.*
Oenin	*Vitis sp.*
Populin	*Populus sp.*
Prulaurasin	*Prunus sp.*

Glucoside	Order or genus
Prunasin	*Prunus sp.*
Quercitrin	*Quercus sp., Fraxinus sp.*
Robinin	*Robinia sp.*
Salicin	*Salix sp., Populus sp.*
Sambunigrin	*Sambucus sp.*
Syringin	*Syringa sp., Ligustrum sp.*

It is interesting to note the occurrence of one and the same glucoside in certain closely related and resemblant species. Willow and poplar, for instance, both belonging to the order *Salicineæ*, and, producing a wood of almost identical structure, contain the same glucoside, salicin. Again, *Prunus* and *Pyrus spp.* both produce amygdalin, while both belong to the order *Rosaceæ*.

It should be noted, however, that many glucosides have been isolated only from the leaves, flowers, or seeds of plants; though others, such as amygdalin, æsculin and salicin are also present in the bark of their derivative species. Systematic research, therefore, might well prove the occasional utility of such substances in identification work, though, in most instances, the wood structure alone will be found to afford a surer basis for diagnosis.

Plant Bases.—These compounds include the *amines*, the *betaines*, the *alkaloids*, and the *purine* and *pyramidine* bases; of which the alkaloids should be briefly considered.

(a) **Alkaloids.**—The importance of these substances in medicine is well known; while the intensely poisonous nature of many of them is equally notorious.[1] Indeed, some woods, such as many belonging to the *Euphorbiaceæ, Anarcardiaceæ* and *Apocynaceæ* are so poisonous (because of the contained alkaloids) that the sap, the saw-dust, or the smoke when burnt, are dangerous and irritating to skin and eyes.

Alkaloids occur in solution in the cell sap[2] of young tissues, as well as in the solid state in older and disused tissue. They may be found abundantly either in the seed, fruit, root or bark, but also in any other part—the woody stem, for example.

Certain alkaloids are said to be restricted to a single genus, or even a single species. In other plants, several alkaloids are found together. Mostly they are insoluble in water and occur as crystalline solids; but a few, such as coniine and nicotine are volatile liquids.

[1] *Vide*, for example "*Poisonous Trees of Central America*," by P. C. Standley, in *Tropical Woods*, Vol. II, No. 9, pp. 3–7. Also various other articles in the same journal.
[2] It is noteworthy that most poisonous trees have a milky latex.

The crystalline types, however, are soluble in alcohol, ether, chloroform, etc. Details as to their extraction and constitution may be obtained from a standard biochemical work. (See Bibliography and Vol. VII, pp. 1–869.)

Alkaloids usually occur in the plant united to an organic acid, such as citric, malic, oxalic, or quinic acid. They are commonly encountered in the following families:—*Anacardiaceæ*, *Apocynaceæ*, *Euphorbiaceæ*, *Leguminosæ*, *Papaveraceæ*, *Ranunculaceæ*, *Rubiaceæ*, and *Solanaceæ;* of these, the first four and the seventh contain many important tree genera, and the *Solanaceæ* a few.

General tests for alkaloids are as follows:—

1. Tannic acid: a white precipitate formed.
2. Brücke's reagent:[1] a white ppte. formed.
3. Phosphotungstic acid:[2] a white ppte. formed.
4. Soln. of iodine in potassium iodine: a brown ppte. formed.
5. Picric acid: a yellow ppte. formed.

As examples of alkaloids found in tree genera, there are *cystine*, from laburnum, *quinine* from *Cinchona spp.*, *strychnine*, *curarine* and *brucine* from *Strychnos spp.*, *berberine* from the barberry, as well as from other isolated genera in several orders (see also Vol. VII).

In conclusion, it should be said that further systematic work would still be required to render the alkaloids really useful to wood science, though a number of specific, or generic, substances from this class are already well known.[3]

SUMMARY OF DIAGNOSTIC METHODS

Three ways of examining an unknown wood sample have now been outlined: the anatomical, the physical and the chemical. Of these, anatomy must be placed first, since it has already been well

[1] 50 grm. of potassium iodide, dissolved in 500 c.c. of water are saturated with 120 grm. of mercuric iodide, and made up to 1,000 c.c.

[2] 50 grm. of phosphotungstic acid and 30 c.c. of conc. sulphuric acid are dissolved in water, and made up to 1,000 c.c.

[3] *Note.*—Before concluding this brief outline of the chemical compounds in plants, that might be found useful in identification and analytical work, reference should be made to an interesting paper entitled "*Recherches sur le determination des bois exotiques colorés d'après les caractères chimiques et spectroscopiques*," by A. Jauffret, in *Ann. Mus. Colonial*, Marseille, 1920: pp. 1–171.

Studies of 17 Indian, 66 Madagascan, 2 Tonkin and 4 French Guiana woods indicated that the chemical and spectroscopic properties of coloured woods may furnish a reliable basis for specific identification. A bibliography of previous work in the same connection is also given.

Briefly, extracts of powdered wood were made in distilled water, alkaline and acidified water, and in 95 % alcohol. Absorption spectra were then studied and certain chemical tests made. Extracts of colouring matter were also made in ether, etc.

These methods deserve attention.

developed and is usually sufficient by itself to enable generic, if not actually specific, identifications to be made. For, broadly speaking, wood structure is of a more specific and reliable nature than such properties as hardness, colour, density, smell, taste, or even chemical constitution. Hardness, for instance, depends to some extent upon growth-rate, the individual or local character of the parent tree, the part of the tree from which the sample was derived, or the physical condition of the sample. So too, do colour and density. Colour, smell and taste, again, may be affected by the relative freshness of the sample; and if the wood be much rotted or charred, they may be altogether absent. Chemical characteristics, too, being both relatively variable from tree to tree, and from one part of a tree to another, are not always to be relied upon. They have, moreover, been insufficiently studied as yet—except in a few species and genera (see p. 478, 493)—to admit of comprehensive or reliable classification, and are also affected by the previous history and present condition of a given sample.

Hence, despite the hindrances of individual variation and inter-specific resemblance, wood anatomy represents the most reliable basis for diagnosis. The total number of woody species, however, runs into thousands, of which several thousand are actually employed commercially at the present day. It is, therefore, essential, even in dealing with a small group such as the indigenous European timbers, to classify woods in accordance with some given scheme.

This necessity has resulted in the construction of what are termed "*keys*"; *i. e.*, schematic aids to identification, based upon the anatomical characters of all the woods (of a given group) under consideration. Such tabulations and their employment in identification work are discussed in the following pages. The construction and use of physical and chemical "keys" would, doubtless, follow along similar lines.

Anatomical "Keys."—It is not practicable to make a single "key" to the identification of all the known commercial woods, or even to the woods of a single rich flora, such as that of India, Africa, or S. America, let us say. The woods of such continents should, therefore, be first grouped geographically, or in accordance with national boundaries, so as to reduce the number to be differentiated. It is for this reason that a foreknowledge of the country of origin of a given sample is often so helpful to the wood structuralist.

Other lines of demarcation may also be devised, however, such as for instance, climatic zones—N. Temperate, Sub-Tropical, etc.; but geographical boundaries seem, on the whole, to be most satisfactory. Yet another way is to group woods systematically by orders and families.

Starting, then, with a relatively small group of commercial woods, such as those of the British Isles, one may proceed to construct either a "macroscopic key" or a "microscopic key."

(a) **Macroscopic and Hand-Lens "Keys."**—As was explained above, a considerable amount of detail may be observed with a low power hand-lens and clean-cut surfaces alone. In addition, physical characters such as colour, density, hardness and odour may be employed, where anatomical distinctions are lacking—unless one is dealing with charred or rotted samples.

Detailed descriptions of the various woods in question having first been prepared (using as many samples of diverse origin as possible, for each species, to allow for individual variation), a rough "key" to their future identification may be prepared, in one of two ways, as follows:—Either a skeleton "key" may be made, which simply segregates the woods into a number of more small tractable groups, and leaves the final decision to a comparison of the observed structure with more detailed descriptions of the woods; or else the final decision is arrived at in the "key" itself, by the further subdivision of groups and the full use of all available diagnostic features. But where the total number of woods to be considered is more than about fifty, say, the latter type of "key" becomes unwieldy, and one is forced to leave small groups of woods for final differentiation, as in the first type of "key," by means of separate, detailed descriptions. In fact, even after a wood sample has been satisfactorily tracked to its apparent source by means of a "key," it is always best to check up on the detailed description at the end, since it is easy to take one false turning which will put one completely upon the wrong track.[1]

[1] *Note.*—A small hand-lens "key" is appended, as example, prepared by the writer in connection with the field identification of European charcoals, which has been tested in practice and found generally useful. On the other hand, atypical specimens will sometimes be encountered for which it was impossible to allow. In such cases, long personal experience is the only sure guide.

This particular "key" consists, for the sake of clearness, of two parts: first, a skeleton "key" proper, involving a series of alternative conditions—between which the investigator has to choose successively, as he examines the unknown sample—, proceeding downwards, in a series of broad steps, until a small group of alternative wood genera is arrived at.

The detailed descriptions, which are arranged alphabetically, and constitute the second

(b) **Microscopic "Keys."**—Finally, it should be repeated here that the ultimate differentiation of very closely similar woods, such as willow and poplar, or alder, hazel and hornbeam, is insecure in the majority of instances without reference to the compound microscope and good micro-sections. Hence, a second "key," involving the use of yet more minute diagnostic features (as already described above), should also be available, in order to settle dubious specimens.

(c) **Detailed Descriptions: Tabulations.**—Detailed descriptions may be prepared either in cursive or tabular form: the latter is very convenient, provided that a given system of abbreviations is first decided upon and strictly maintained. *Standardisation of terms,* of whatever kind, is, in fact, one of the prerequisites of wood-structural work, and international efforts are now being made in that direction. But if such standards do not exist, then the individual worker should choose and adhere to his own terms throughout all his investigations, unless he is to be involved in much time-wastage and confusion.

A tentative schedule of convenient abbreviations for the recording microscope characters will be found at the end of this article. (See *Appendix II.*)

part of the "key," are then referred to, and the structure of the sample in hand compared with each in turn, until the closest agreement is obtained. Subsequently, however, the process should be repeated as safety check. (See *Appendix I.*)

"KEY" TO EUROPEAN WOODS

APPENDIX

I. A Hand-lens "Key" to 35 European Woods.

	Genus
I. Pores Absent.	
A. VERTICAL RESIN DUCTS PRESENT.	*Pinus.*
B. VERTICAL RESIN DUCTS ABSENT.	*Juniperus* *Taxus.*
II. Pores Present.	
A. RING POROUS. (PORES MOSTLY AND/OR CONSPICUOUSLY FORMING A WELL-DEFINED RING.)	
(1) *Presence of large rays conspicuous to the eye.*	
a. Larger pores conspicuous to the eye.	*Morus.* *Quercus.* *Rosa.*
b. Larger pores almost or quite invisible to the eye, but distinct with lens.	*Laburnum.* *Ulex.*
(2) *Absence of large rays conspicuous to the eye.*	
a. Pores pretty uniform in size	[1]*Prunus.*
b. Transition from large to small pores gradual.	*Castanea.* [1]*Juglans.*
c. Transition from large to small pores relatively abrupt	*Fraxinus.* *Ulmus.*
B. DIFFUSE POROUS. (PORES DIFFUSED THROUGHOUT THE WOOD, AND A CONSPICUOUS PORE-RING ABSENT.)	
(1) *Presence of large rays conspicuous to the eye.*	
a. Large rays very conspicuous, but irregularly distributed. Fine rays present.	
1. Rays of two distinct sizes, very broad and very fine	[1]*Alnus.* *Corylus.* *Carpinus.*
2. Rays of all sizes, from very broad to fine.	*Fagus.*
b. Large rays fairly conspicuous, many, and regularly distributed. Fine rays absent.	*Platanus.*
(2) *Absence of large rays conspicuous to the eye.*	
a. Small rays clearly visible with hand-lens.	
1. Pores clearly visible with hand-lens.	
i. Rays broad and conspicuous with lens.	*Acer.* *Hedera.* *Sambucus.*
ii. Rays relatively fine with lens.	*Betula.* *Cornus.* [1]*Juglans.* *Tilia.*

Note.—Woods possessing pores are derived from *Angiosperms* ("hardwood" trees) those lacking pores are from Gymnosperms ("softwood" trees).

([1] All descriptions refer to transverse sections only, viewed by a person with sight of average quality, using a lens magnifying × 6–8 linear. For reference to illustrative plates, see below.) Genera marked thus are entered in more than two places in the "key," owing to individual variability of structure. Thus *Juglans sp.* may either appear ring-porous or diffuse-porous, according to growth conditions.

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	Genus
2. Pores scarcely visible with hand-lens.	
i. Rays fairly broad and conspicuous with lens...	*Ilex* [1]*Prunus.*
ii. Rays relatively fine with lens................	*Crataegus.* *Pyrus.* [1]*Rhamnus.*
b. Small rays almost or quite invisible with hand-lens.	
1. Pores clearly visible with hand-lens.	
i. Pores pretty uniform in size and distribution, and often forming wavy tangential pattern...	*Olea.* *Populus.* *Salix.*
ii. Pores typically becoming less distinct toward end of ring. Patterning absent..............	*Alnus.* *Viburnum.*
2. Pores almost or quite invisible with hand-lens.	
i. Pores pretty uniformly distributed....	*Aesculus.* *Buxus.* *Euonymus.*
ii. Pores arranged in oblique radial streamers.....	[1]*Rhamnus.*

DETAILED CHARACTERS OF INDIGENOUS EUROPEAN WOODS, AS SEEN WITH A HAND-LENS UPON A CROSS-SECTIONAL SURFACE

Acer sp. (Maple and Sycamore).	Pores small, relatively few, circular in appearance, mostly solitary, and uniformly distributed. Absence of any definite marginal line of pores. Wood fine-textured in general appearance. Rays all of one kind, lustrous, and very distinct with lens.
Aesculus sp. (Horse-Chestnut).	Pores very minute and many, mostly grouped, and commonly decreasing in number toward the end of the growth-ring. An ill-defined marginal line or pores (of same size as the rest) sometimes visible. Wood fine-textured in general appearance and whitish. Rays all of one kind and very fine; scarcely visible with hand-lens and very numerous.
Alnus sp. (Alder).	Pores very small and many, mostly in short radial groups and typically uniformly distributed. An indistinct marginal line of very small pores often present. Wood fine-textured in general appearance. Rays of two kinds; very conspicuous, but only occasional, broad rays, easily visible to the naked eye, and very fine rays, almost invisible with the lens.
Betula sp. (Birch).	Pores very small, relatively few, uniformly distributed, typically in short radial groups of 2–4, and presenting a lenticular aspect. Wood fine-textured in general appearance. Rays of one kind only, fine and fairly distinct with the hand-lens, often sinuous. Pith flecks common.
Buxus sp. (Box).	Pores many, very minute indeed, practically invisible with the lens, and uniformly distributed, except that edges of rings are often lacking in pores.

Note.—Woods possessing pores are derived from *Angiosperms* ("hardwood" trees) those lacking pores are from *Gymnosperms* ("softwood" trees).

All descriptions refer to transverse sections only, viewed by a person with sight of average quality, using a lens magnifying × 6–8 linear. For reference to illustrative plates, see below.

[1] Genera marked thus are entered in more than one place in the "key," owing to individual variability of structure. Thus *Juglans sp.* may either appear ring-porous or diffuse-porous, according to growth conditions.

Wood dense, especially at the ring margin, and fine-textured in general appearance.
Rays of one kind only, very fine indeed and invisible with a hand-lens.

Castanea sp. (Sweet-Chestnut). A definite marginal ring of large pores visible to the unaided eye, but also many smaller ones (distinct with lens) trailing off in obliquely radial lines between the main pore rings, and diminishing in size.
Wood coarse-textured in general appearance.
Rays of one kind only, fine, but just visible with lens.

Carpinus sp. (Hornbeam). Pores very small, relatively few (fewer and larger than in *Alnus* and *Corylus*), commonly in short radial groups (shorter than in *Corylus*), usually decreasing in size towards the end of the ring. Absence of a definite line of pores at the ring margin.
Wood fine-textured in general appearance.
Rays of two kinds; large rays, easily conspicuous to the naked eye and fairly many, (often being pretty evenly distributed), and fine rays, relatively few, but just distinct with the hand-lens.

Cornus sp. ("Dogwood?")[1] Pores very small and many, sometimes decreasing in number at end of growth-ring, and mostly solitary. A marginal line of small pores usually present, though not distinct. (Pores more circular in form and fewer than in *Pyrus*.)
Wood fine-textured and dense in appearance.
Rays of one kind only, fine, but distinct with hand-lens.

Corylus sp. (Hazel). Pores very small and many, but typically decreasing toward end of the ring, and often tending to form radial strings. A definite marginal line of small pores often present. (Pores fewer than in *Alnus*, but more than in *Carpinus*, and being typically in radial groups can usually be distinguished therefrom.)
Wood fine-textured in general appearance.
Rays of two kinds; fairly frequent large rays, irregularly spaced, conspicuous to the naked eye, and fine rays, scarcely visible with hand-lens.

Crataegus sp. (Hawthorn). Pores very small, very many, mostly solitary, as in *Pyrus*, and considerably aggregated towards the beginning of the growth-ring (more so than in *Pyrus*); otherwise generally diffused. *Sometimes a line of small pores evident at ring* margin, but not usually noticeable.
Wood dense and fine-textured in general appearance.
Rays all of one kind, fine, but distinct with hand-lens.

Euonymus sp. (Spindle-tree). Pores very minute, very many, just visible with lens, decreasing towards end of the growth-ring, and a distinct marginal line of slightly larger pores present at beginning.
Wood fine-textured in general appearance.
Rays all of one kind, very fine and often invisible with lens.

Fagus sp. (Beech). Pores small and very many, aggregated towards the beginning of the ring, decreasing in size towards the end of the year, and often in minute tangential groups. (Pores typically a little smaller than in *Platanus*.)
Wood fine-textured in general appearance.
Rays of two kinds; frequent large rays, easily visible to the naked eye, irregularly distributed, and finer rays also distinct with hand-lens. (Large rays fewer than in *Platanus*.) See also tangential longitudinal section.

[1] Properly called *dagwood*, or else *gad-treow* (= goad-tree), from the early usage of the wood.

Fraxinus sp. (Ash).
Pores not very many, two distinct sizes, mostly aggregated into a distinct, clear-cut zone of large pores at the beginning of the ring, visible to the naked eye.
Apart from the pore-rings are a few scattered, smaller pores, often in groups of two or more.
Wood coarse-textured in general appearance.
Rays of one kind only, fine, clear-cut, and distinct with hand-lens.

Hedera sp. (Ivy).
Pores very small and many, typically packed close together in continuous, parallel tangential bands, 3 or 4 deep, and gradually decreasing in size a little across the ring. (General appearance resembling that of *Prunus*, but pores more numerous, and less ring-porous in type.)
Wood fine-textured in general appearance.
Rays of one kind only, scarcely visible to naked eye, but mostly broad and conspicuous with hand-lens; of varying width.

Ilex sp. (Holly).
Pores many, very minute indeed, and typically aggregated in small radial strings.
Wood fine-textured in general appearance, and dense.
Rays of one kind only, and (though diffuse in appearance) typically broad and conspicuous with hand-lens.

Juglans sp. (Walnut).
Pores commonly aggregated in an ill-defined zone at the beginning of the growth-ring, (easily visible to the eye), with others diffused throughout the wood; relatively few in number, and rapidly decreasing in size (as in *Castanea*). The smaller pores sometimes trail across the ring in loose radial lines. (*Juglans* is on the border-line between the more truly ring-porous and more truly diffuse-porous woods.) Pores of medium size, slightly elongated, mostly single.
Wood relatively coarse-textured in general appearance.
Rays of one kind only, fine, but quite distinct with lens.

Juniperus sp. (Juniper).
Pores absent (being a Conifer). Vertical resin ducts absent.
Wood denser than that of *Pinus*, and finer textured. Heartwood light red in colour. Appearance slightly more homogenous than that of *Taxus*.
Rays just visible with a hand-lens; of one kind only.

Laburnum sp. (Laburnum).
Small to medium-sized pores forming a distinct marginal ring, visible to the eye. Between the pore rings occur distinct short, broad, wavy bands of smaller pores. The change from large to small pores is relatively abrupt.
Wood coarse-textured in general appearance, but mainly dense.
Rays of one kind only, being visible to the naked eye, and large and uniform in a hand-lens view.

Morus sp. (Mulberry).
Pores large, visible to the naked eye, and the larger ones aggregated to form a definite marginal ring (pores bigger than in *Laburnum*); the remainder gradually decreasing in size, and uniformly distributed in clusters of two or three, but sometimes forming wavy tangential bands. (Not so broad or continuous as in *Ulmus* or *Laburnum*.)
Wood fairly dense, but of coarse texture.
Rays of one kind, mostly distinct to naked eye, but of varying width under hand-lens.

Olea sp. (Olive).
Pores very small and fairly many, typically in small radial groups of two or more, relatively uniform in size, and commonly forming almost straight, parallel tangential lines.
Wood fine-textured in general appearance.

Rays of one kind only, and scarcely visible with hand-lens.

Pinus sp. (Pine).

Pores proper absent (being a Conifer.)
Vertical resin canals common, and conspicuous as medium-sized whitish spots, or "fuzzy pores," and often in loose tangential lines, or scattered here and there about the wood. (Large pores smaller than in *Ulmus*.)
Wood light, coarse-textured and heterogeneous in general appearance.
Rays of one kind only, very many, and just visible with hand-lens.

Platanus sp. (Plane).

Pores very small, very many, evenly diffused, and mostly in small bunches betwixt the rays; (a little smaller than in *Fagus*).
Wood fine-textured in general appearance.
Rays all of one kind, visible with naked eye, and appearing broad and uniformly distributed beneath the hand-lens, but some a good deal narrower than others.

Populus sp. (Poplar).

Pores very small and many, decreasing in size towards the end of the growth-ring, often in twos or threes; sometimes a line of pores noticeable on the ring margin. Pores often in loose oblique and wavy lines. (More so than in *Salix*.)
Wood light and fine-textured in general appearance.
Rays of one kind only, very fine, indistinct with lens.

Prunus sp. (Cherry, Plum, etc.).

Pores very small, few to many, mostly grouped, often arranged in short tangential or wavy lines, and considerably larger and aggregated at the beginning of the growth-ring to form a distinct line of small pores 2–3 deep; otherwise generally diffused. Pith flecks occasional.
Wood fine-textured in general appearance, and dense.
Rays of one kind only, fairly broad, conspicuous with hand-lens, and typically swollen at the ring margin. (Broader than in *Crataegus*.)

Pyrus sp. (Apple, Pear, etc.).

Pores very small and very many, mostly solitary, sometimes decreasing in size (though not in number) towards the end of the ring. (More uniformly distributed than in *Crataegus*.)
Wood fine-textured in general appearance and dense.
Rays of one kind only, just visible with hand-lens, and numerous. (Usually less distinct than in *Crataegus*.)

Quercus sp. (Common Oak).

Very large pores, forming a well-marked marginal ring, and easily visible to the eye. Much smaller pores also present, usually grading off rapidly in size, as diffuse radial streamers between the main pore rings.
Wood dense (apart from pores), but of a coarse texture in general appearance.
Rays of two kinds; occasional large and conspicuous rays, irregular in size and distribution, easily visible to naked eye, and very fine rays invisible with hand-lens.

Rhamnus sp. (Buckthorn).

Pores very minute indeed, many, but collected entirely into lines and oblique radial streamers.
Growth-rings often rather indefinite; but fine marginal pore rings of moderately continuous nature are present, and between these are many wavy oblique radial streamers.
Wood dense and fine-textured in general appearance.
Rays of one kind only, fine, and scarcely visible with lens.

Rosa sp. (Rose).

Pores relatively few and of two distinct sizes, the larger ones being arranged in a definite marginal ring, usually only one deep, with the remainder rapidly decreasing in size, and uniformly diffused. The smaller pores sometimes form loose

tangential lines, one deep, but mostly solitary. (Compare with *Morus*.) The larger pores are often crowded together and elongated radially.
Wood of fairly coarse texture in general appearance.
Rays of one kind but variable width, mostly visible to the naked eye. (Rather more conspicuous than in *Morus*.)

Salix sp. (Willow). Pores very small, very many, mostly solitary, and usually uniform in size and distribution, except that slight graduations *may occur with seasonal changes, and a single clear line of* small pores is rarely present, marking the margin of the growth-ring. Short, wavy tangential groupings of the pores are less marked than in *Populus*.
Growth-rings often indefinite, and frequently very broad.
Wood light and of a porous, though homogeneous texture. (Much resembling *Populus*.)
Rays of one kind only, very fine and invisible with hand lens.

Sambucus sp. (Elder). Pores very small and many, mostly in small groups or wavy tangential lines, and slightly aggregated at the beginning of the growth-ring to form a distinct zone; decreasing in size a little towards end of ring; otherwise evenly distributed.
Wood fairly dense and fine-textured in general appearance.
Rays conspicuous with a hand lens (as in *Prunus*, but not swollen).

Taxus sp. (Yew). Pores absent *(being a conifer)*.
Vertical resin ducts absent.
Wood fairly dense. *(Relatively homogeneous and fine-textured in comparison with *Pinus*, and rather heavier than *Juniperus*.)*
Heartwood light-red in colour.
Rays of one kind only, many, but practically invisible with hand lens.

Tilia sp. (Lime). Pores very small and many, uniformly distributed, and mostly in small groups. A fine marginal line of small pores of elongated form usually present.
Wood fine-textured in general appearance.
Rays of one kind only, fine but distinct with hand lens, and slightly swollen at the ring margin—where visible.

Ulex sp. (Gorse). Pores very small to minute, relatively few, and restricted to oblique, criss-crossed, wavy bands, tending to aggregate at the beginning of the ring.
Pores uniformly decreasing in size across the ring, and sometimes forming a fairly distinct marginal line. (More clearly visible than in *Rhamnus*.)
Wood of fairly fine texture in general appearance.
Rays all of one kind, but variable width; mostly visible to naked eye, and broad and conspicuous with hand lens.

Ulmus sp. (Elm). Medium-sized pores, visible to naked eye, usually forming a well-defined ring 1–3 deep. Much smaller pores also present in tangential and wavy bands (distinct with a hand lens) betwixt the main pore rings, generally three or more pores broad. (The change in size from large to small pores is usually more sudden in *Ulmus Campestris* than in *Ulmus montana*. Large pores larger than in *Laburnum*.)
Wood of fairly coarse texture in general appearance.
Rays of one kind only, fine, but conspicuous with a hand lens. (Rays much finer than in *Laburnum*.)

Viburnum sp. (Guelder etc.). Pores very small and many, mostly solitary, decreasing in size a little toward the end of the growth-ring, and sometimes

forming a fairly definite zone at the ring margin; otherwise uniformly distributed.
Wood dense and fine-textured in general appearance.
Rays of one kind only, almost invisible with a hand lens. (Rays rather finer than in *Pyrus*, and pores smaller.)

Explanation of the "Key"

In the foregoing descriptions only four features of wood anatomy were referred to, for sake of simplicity.

Viz., 1. The "*pores*," or conducting vessels as seen in cross-section. (Hardwoods only.)

2. The *storage*, or medullary, *rays*, which run radially, from the centre of the tree outwards.
3. The *resin canals* which may look like medium-sized "fuzzy" pores. (Softwoods only.)
4. The relative *density and texture* of the wood, as judged superficially from air-dried specimens.

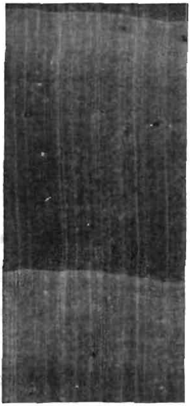
Particular instances of these characters are illustrated by *Plate I* as explained below.

Diagnostic character	Numbers of illustrative photos
"Pores absent." (But resin ducts present.)	I
"Pores present"	II—VI
"Ring porous." (And pores of two distinct sizes)	II, III
"Diffuse porous"	IV–VI
"Presence of large rays conspicuous to the eye" (one shown)	V
"Large rays very conspicuous. Fine rays also present"	V
"Small rays clearly visible with hand lens"	III, IV
"Small rays almost or quite invisible with hand lens"	II, V, VI
"Pores clearly visible with hand lens"	IV, VI
"Pores almost or quite invisible with hand lens"	Smaller than in V
"Pores very small and many"	V, VI
"Pores relatively few"	II, IV
"Pores mostly solitary"	IV
"Pores uniformly distributed"	IV, VI
"Pores decreasing in number toward the end of the growth-ring"	II, V
"A distinct line of small pores present at the ring margin"	V
"Pores typically decreasing in size near the end of the growth-ring." (But diffuse-porous type)	V, VI
"Pores mostly in short radial groups"	V
"Pores arranged in radial, or oblique, lines or streamers"	II
"Small pores arranged in short wavy, tangential lines, or bands"	III, (VI)
"Sharp transition from large to small pores". (Common to ring-porous type)	II, III

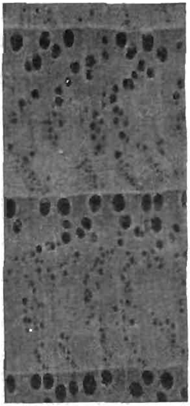
II. Schedule of Microscopic Characters and Abbreviations.

Annual Rings.
Present +, absent −.
Ring Porosity.
Well defined +, dubious ±, absent −.
Vessel Size.
Mean diam. of 100 vessels expressed in $\mu\mu$. Also range.
Radial Groups.
Typical number of vessels in a single group. (State range.)

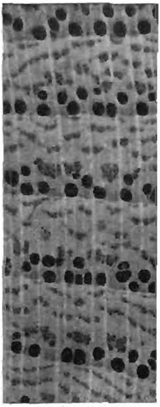
STRUCTURE OF WOODS



(1) Scotch Pine
(*Pinus sylvestris*)

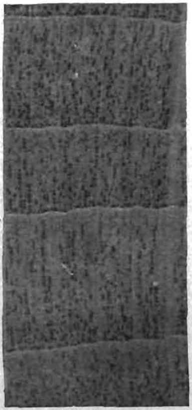


(2) Sweet Chestnut
(*Castanea vesca*)

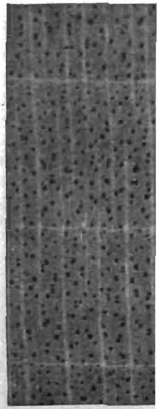


(3) Common Elm
(*Ulmus campestris*)

(4) Field Maple
(*Acer campestris*)



(5) Hazel
(*Corylus avellana*)



(6) Black Poplar
(*Populus nigra*)

All are transverse sections. × 10 linear, as viewed under surface illumination.

Vessel Pattern.
Well defined +, indefinite ±, absent −.
Radial arrangement R, tangential T, wavy W, oblique O, parallel //.

Vessel Number.
In diffuse porous woods, state the average number *per* unit area.

Vessels Solitary.
More than half of vessels solitary shown as +, about half ±, less than half −.

Spiral Thickenings.
Presence or absence in vessels or tracheids denoted as + or −.

End Perforations to Vessels.
Multiple M, Scalariform S, Simple O.

Gum and Tyloses.
Presence denoted as G. and T. respectively. Abundant ++ absent −.

Fibre Arrangement.
Definite radial arrangement as +, lack of arrangement as −.

Fibre wall.
Width of lumen greater than (double thickness) fibre wall defined as +, equal ±, less −.

Septate Fibres.
Presence or absence denoted as + or −.

Parenchyma.
B....... Broken bands.
C....... Continuous bands.
T. ..... Terminal, along ring margin.
S....... Scattered (solitary or diffuse).
V....... Vasicentric (around vessels).
VT...... Vasicentric, with tangential wings.
+.. ... Parenchyma abundant, ± moderate quantity.
−...... Parenchyma not noticeable.

Ripple Marks.
Presence or absence of storied rays denoted as + or −.

Ray Type.
Uniseriate........ U.
Diffuse.......... D.
Aggregate........ A.
Compound....... C.

Ray Number.
Mean number of rays crossing a unit length of a growth-ring.

Ray Height.
Typical height of fully formed rays, counted in cells. (Also state max.)

Ray Width.
Typical number of cells broad (state range) as seen in a trans. section.
Rays wider than large vessels +, about equal ±, narrower −.

Marginate Rays.
Presence or absence denoted as + or −; doubtful cases as ±.

Ray Contents.
F..... Full.
L..... Lining walls.
G..... Globular.

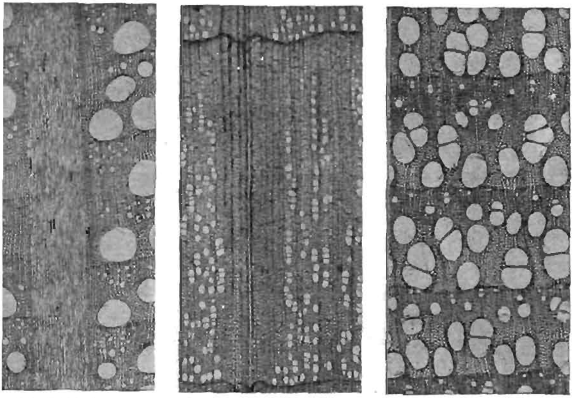
Ray Pits (Simple).
+.... Conspicuous.
−.... Inconspicuous.
S..... Slitlike.
R..... Round.

Ray Tracheids.
Present +, absent −.

Reticulate Thickenings.
Presence in ray tracheids denoted as +, absence as −.

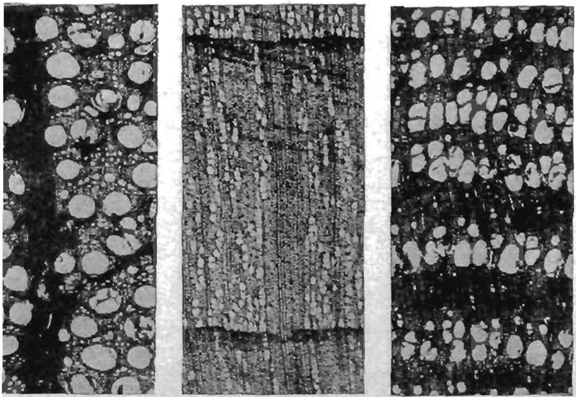
STRUCTURE OF WOODS

A *B* *C*



Common Oak (*Quercus sp.*) | Hazel (*Corylus sp.*) | Ash (*Fraxinus*) *sp.*)

a *b* *c*



low: Roman charcoals (*a*, *b*, *c*) for comparison with the woods (*A*, *B*, *C*.) shown
:. All are transverse sections, × 30 linear, as viewed by transmitted light. Note
la and *Cc* are samples with very narrow growth rings.

Resin Ducts.
V..... Vertical.
H..... Horizontal.
—..... Absent.

Crystal Cells.
Presence or absence denoted as + or − (R...in rays, P...in parenchyma).

Remarks.
Any peculiarities, such as intraxylary phloem, pith flecks or stone cells.

III.—The Identification of Particles of Powdered Wood and Charcoal.

The certain identification of microscopic particles of wood or charcoal, such as may be met with in sawdust, synthetic lumber, archaeological soil and hearth deposits, fuel blocks, gunpowder, or commercial decolourizing charcoals, is a difficult matter: often it is impossible. Even where one can assume that only a single wood is involved, the largest fragments are generally too minute to section, and, though sectioned, would not include sufficient tissue to permit of identification along ordinary lines.

At best, the nature of fragments a few millimetres broad and long can only be guessed at by one who knows his woods intimately.

In a paper entitled "*The Identification of Norit and other Wood Charcoals*," by H. G. Tanner, in Industrial and Engineering Chemistry, 1925, vol. 17, pp. 1191–1193, what appears to be a reliable method for identifying charcoal dust is, however, described.

In an attempt to determine the constitution of a highly activated decolourizing charcoal, called "*Norit*," introduced about 1911, it was found that minute particles of cell wall substance (apparently from the walls of conducting vessels or tracheids), termed "identification particles," were always present. These particles, examined under a high power of the microscope (× 500 say), were seen to be perforated by numerous minute pits, the relative size, frequency and pattern of which were found to constitute a generic, if not also a specific, character. In this way, "*Norit*" was decided to have been made from Birch wood (*Betula occidentalis?*).[1]

The mere presence of (normal) woody tissues in a soil deposit, or other finely divided sample, may, of course, be tested for with phloroglucinol followed by conc. hydrochloric acid—giving a fine red colour when lignin is present.

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[1] *Note.*—The writer has not yet had occasion to employ this method himself, and a considerable amount of systematic work would first be required before it could be put into practice; but judging by what he has seen of cell-wall pitting in woods, it should prove satisfactory.

One possible objection occurs; namely, that the size, form and arrangement of such pits undoubtedly varies to some extent within a given species, or even from part to part of a single cell wall. Moreover, a considerable amount of shrinkage takes place in wood when it is charcoaled. In incompletely charred specimens, therefore, the mean diameter of such pits might be greater than in specimens which had been treated for a longer period of time.

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THE PECTIC SUBSTANCES

BY H. W. BUSTON, PH.D., D.I.C.

Among the large variety of substances which together constitute the cell-wall material of plants, there is one group, apart from the celluloses, which has of late years assumed a definite commercial importance. This group comprises the substances known collectively as the *pectins*. Pectic substances have been recognised as constituents of the cell-wall since 1824, when Braconnot isolated a number of pectic products from fruits, fleshy roots, and other plant materials. He was able to show that the formation of jellies by certain fruit juices in the presence of sugar was due to the pectin in the juice. This ability to form jellies with sugar is the basis of the main industrial use of pectin preparations; as will be seen later, however, the pectins enter into many other industrial problems, such as those met with in the fermentation and textile industries, and problems connected with the storage of fruit and vegetables.

Investigations on the chemistry of the pectins, and on their distribution in plant substances, were recorded from time to time throughout the last century, but only during recent years has any complete understanding of the chemistry of these substances been approached, and their industrial use rendered possible. A considerable amount of confusion also has arisen through lack of any standard nomenclature for the different members of the group; thus many of the early papers lose much of their value on this ground. The nomenclature tentatively agreed upon by the Pectin Symposium of the American Chemical Society in 1925, and given below, seems the most satisfactory system, and is now generally used, but there are still some authors who do not conform thereto. Thus Branfoot, in an important monograph (1929) revives the old term "pectose" in place of "protopectin"; and there is no doubt that the "pektinsaure" of Ehrlich is not true "pectic acid," but one of the partly esterified pectic acids ("pectinic acid") of the American system.

Nomenclature.—The system most widely adopted recognises the following groups of pectic substances:

(1) *Protopectin:* an insoluble material, present in the cell-wall, capable of conversion into soluble pectin by hydrolysis. In protopectin, the pectin is probably associated or combined with cellulose and metallic ions. The names pectose, "insoluble pectin" and pectocellulose have also been applied to this substance.

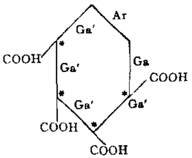
(2) *Pectin:* a soluble substance, or group of substances, present in the cell-wall and also in the cell-contents; liberated from the cell-wall by gentle hydrolysis, and consisting of the methyl esters of pectic acid. The term "pectin" has been used by some workers to indicate only the fully esterified form of pectic acid, containing 11.76% methoxyl; the intermediate compounds in which the carboxyl groups are only partly esterified being known as "pectinic acids." The term "pectinogen" has also been used, indicating a mixture of pectin and pectinic acids, with possibly some soluble pectates. The soluble pectins are characterised by their ability to form jellies with sucrose in the presence of acids.

(3) *Pectic acid:* the insoluble, acid material, derived from pectin or pectinic acids by complete de-esterification; it forms the basal molecule of all the pectic substances. It is incapable of forming sucrose-acid jellies.

(4) *Metapectic acid:* soluble substances, probably representing stages in the degradation of pectic acid to simple sugars and sugar acids. Hardly true pectic substances at all.

Chemical Constitution of Pectic Substances.—(a) *Pectic Acid.*—While there is still some difference of opinion as to its chemical structure, there is general agreement that the parent substance of all pectins is pectic acid. This compound is readily prepared from any pectic material, and is of constant composition from all sources. It is most easily obtained by treating pectin, prepared by extraction of suitable plant tissues by one of the methods described below, with dilute sodium hydroxide solution, whereby sodium pectate is formed; from this, the free acid is liberated by addition of excess of hydrochloric acid. (Full details of this preparation are given in a subsequent section.) Pectic acid forms a white powder, practically insoluble in water, but readily soluble in alkali; it has no reducing properties, but readily undergoes hydrolysis, yielding a mixture of reducing sugars in which arabinose and galac-

tose may be detected. The work of Ehrlich (*Chem. Ztg.*, 1917, **41,** 197) and others proved that residues of galacturonic acid form a fundamental part of the molecule, and in fact make up the greater part of the pectic acid structure. Calculations based upon estimations of furfural yield (from arabinose and galacturonic acid residues), carbon dioxide (from galacturonic acid), and mucic acid (from galactose and galacturonic acid) led Nanji, Paton and Ling (*J. Soc. Chem. Ind.*, 1925, **44,** 253T) to the conclusion that pectic acid contains the anhydrides of 1 molecule of arabinose, 1 molecule of galactose, and 4 molecules of galacturonic acid. They put forward the suggestion that these residues are combined in the form of a six-sided ring, according to the accompanying diagram:—



Ar = Arabinose residue
Ga = Galactose residue
Ga' = Galacturonic acid residue
* = Linkage other than 1:6

Such a molecule would have an empirical formula $C_{35}H_{50}O_{33}$, which is in close agreement with that deduced by various workers from the results of elementary analyses, *e. g.*, Schryver and Haynes, (*Biochem. J.*, 1916, **10,** 539)—$C_{17}H_{24}O_{16}$; von Fellenberg (*Biochem. Z.*, 1918, **85,** 118)—$C_{70}H_{104}O_{68}$. The galacturonic anhydride content of such a molecule would be 70.5%, and the furfural yield (from the arabinose and galacturonic acid residues) approximately 20%; the calcium salt of the acid, which is tetrabasic, would contain 7.45% Ca. All these values are in good agreement with those actually obtained for pectic acid, and accepted as characteristic (p. 528).

The formula suggested by Nanji, Paton and Ling has not met with universal acceptance. For instance, von Fellenberg, from a study of furfurals yielded by pectic acid, was led to conclude that a methyl pentose residue was present, and further considers that the acid is octabasic; the above formula makes no provision for the presence of a methylpentose, and it may be pointed out that no such compound was actually isolated, in the free state, by von Fellenberg.

More important are the views of Ehrlich, whose "pektinsaure" was originally isolated in the form of a calcium-magnesium salt from sugar-beet residues. The extraction was carried out by heating the residues with water under pressure, and precipitating the pectic substances from the extract by addition of alcohol; an araban (tetra-anhydro-arabinose) remained in solution. Analysis of the precipitated acid led Ehrlich to the conclusion that it was formed by the condensation of 4 molecules of galacturonic acid, 1 molecule of galactose, 1 molecule of arabinose, 3 molecules of acetic acid, and 2 molecules of methyl alcohol. A preparation similarly obtained from flax was found to contain only 2 molecules of acetic acid, but contained also 1 molecule of xylose. Thus the constitution assigned by Ehrlich to pectic acid differs from that suggested by Nanji, Paton and Ling in providing for the presence of acetic acid, methyl alcohol, and (in some cases) xylose. One important difference in properties exists between the pectic acid described by Ehrlich and "pectic acid" as usually understood; Ehrlich's product is soluble in water, whilst that obtained by the method outlined above is insoluble. It would seem that this fact, and the fact that Ehrlich's acid yields methyl alcohol, indicates that the substance is not a true pectic acid, but is one of the partly esterified pectinic acids, which are known to contain methyl groups. No other investigator has reported the presence of acetic acid in the molecule, nor has the presence of xylose been suggested; since xylans are frequently associated with pectins in the cell-wall, especially in partly lignified tissues, it seems possible that the xylose in the pectic acid of flax was derived from that source.

Again, Henderson (*J. Chem. Soc.*, 1928, 2117) isolated from flax a "pectic acid" containing no arabinose; while Bowman and McKinnis (*J. Biol. Chem.*, 1930, **52**, 1209) obtained from apples a digalacturonic acid, and from oranges an arabino-galacturonic acid, and suggested that pectic acid was simply a polymerised form of these acids, and that a number of types of pectin, based upon these nuclei, might exist in the plant.

On the whole, it seems that the Nanji, Paton and Ling formula gives the most satisfactory representation of the structure of pectic acid, and, in the absence of further evidence, may be taken as a convenient working hypothesis. The existence of other pectic acids in certain exceptional cases is not precluded.

(*b*) *Pectin*.—Pectin and the pectinic acids (pectinogen) comprise a group of substances in which the four carboxyl groups in the pectic acid molecule are more or less completely esterified, in the form of methoxyl groups. A neutral pectin, having all four carboxyl groups esterified, would contain 11.76% methoxyl; the tri-, di-, and monomethyl esters would contain respectively 8.94%, 6.04% and 3.06% methoxyl. Cases have been recorded of the isolation of pectins having a methoxyl content corresponding with these values, but it is unlikely that such products were more than chance mixtures of the various partly esterified compounds. As usually prepared, the pectins have a methoxyl content lying between 3% and 9%; isolated instances are recorded in which the methoxyl value approached the 11.76% required by neutral pectin, but, in general, the values do not exceed 9%—approximately that required by the trimethyl ester of pectic acid. For this reason, it has been suggested (Norris, *Biochem. J.*, 1926, **20**, 993) that this represents the degree of esterification in the tissue, and that the fourth carboxyl group is involved in combination with cellulose or with metallic ions in the cell-wall. Other workers consider that natural pectin is the tetramethyl ester, and that the products isolated owe their lower yield of methoxyl to contamination with about 25% of non-pectic material.

The ester groups in the pectins are removed extremely readily by the action of weak alkali, with the formation of the pectate of the alkali metal. The reaction is complete in a few minutes at ordinary temperatures. Towards acids, the ester groups are more stable, but boiling with acid stronger than $N/75$ results in hydrolysis. Hydrolysis is also promoted by certain enzymes occurring in plant tissues.

(c) *Protopectin*.—(Pectose).—Protopectin is the water-insoluble complex present in the cell-wall of plants, from which soluble pectins are derived by hydrolysis. In protopectin, the pectin is combined with varying amounts of cellulose, this latter substance apparently replacing, more or less completely, the methyl groups of pectin. No method is known whereby protopectin can be isolated in an unchanged condition. The most successful attempt at isolation was that of Sucharipa (*J. Amer. Chem. Soc.*, 1924, **46**, 145), who, from the white pith of lemon peel, repeatedly extracted all substances soluble in water, alcohol and ether, and then removed

cellulose by means of Schweitzer's reagent. The residue, after thorough washing in acetic acid and water, was considered to be "pure protopectin." The method is not applicable to all tissues (*e. g.*, apple), since treatment with Schweitzer's reagent is found to dissolve the whole of the tissue, including the protopectin. The "pure protopectin" of Sucharipa yielded, on extraction with various solvents, soluble pectins whose methoxyl content varied from 11.7% to 2.05%, this last fraction being most difficult to extract, and probably owing its resistance to the replacement of most of the methyl groups of pectin by cellulose residues. Protopectin must therefore be regarded as a substance of variable composition; besides cellulose residues, inorganic ions are probably always present, especially calcium and iron.

Distribution of Pectic Substances in Plant Tissues

The pectic substances are of the widest distribution throughout plant tissues, being in fact present in most, if not all, parts of the higher plants. They are found to the greatest extent in fruits, such as apples, citrus fruits, currants, etc., and in fleshy roots, such as turnips, carrots and beet. The commercial pectins are mainly obtained from these sources, especially where such materials occur in large amounts as by-products of other industries, *e. g.*, citrus fruit residues in Italy, Florida, etc., residues from cider-pressing, beet sugar manufacture, etc. The accompanying table gives some idea of the amount of pectic substances present in a number of typical plant tissues.

Source	Total pectins, as % dry tissue	% moisture in fresh tissue
Apple (average section)	17.5	87
Orange (white pith)	38.5	76
Orange (average section)	24.5	85
Lemon (peel)	35.5	77
Carrot	22.5	90
Turnip	18.0	90
Onion	13.5	...
Leaves (various)	4–7	70–87

In general, pectins are most abundant in non-lignified tissues, although they have been detected in timbers; in the lignified parts of the plant they are replaced to some extent by hemicelluloses, which are in some ways of similar constitution.

With regard to the individual pectic substances present in plants, these are of all types. The cell-sap contains soluble pectins, with possibly some soluble pectates. The cell-wall contains a relatively large amount of protopectin, which is laid down as an incrustation on the cellulose membrane constituting the first partition between the cells after division. Finally, the middle lamella, the cementing material binding the cells together, seems to be composed largely of calcium pectate.

Extraction of Pectic Substances

(1) **Qualitative.**—Since, as has been pointed out above, the different classes of pectic substance vary considerably in their solubility, it follows that the use of different solvents will result in the extraction of mixtures of pectins of varying composition; appropriate solvents may therefore be chosen according to the class of pectin which it is desired to prepare.

In all cases, prior to extraction of the pectin, the material should be thoroughly washed (which treatment will, however, extract any soluble pectin), and extracted with alcohol and ether; the residue should then be dried in the air and ground to a uniform powder.

For the extraction of total pectic material, the most useful solvent is 0.5% ammonium oxalate. The powdered material is treated with excess of this reagent, and heated on a boiling water-bath for several hours. Almost the whole of the pectin is removed within 12 hours, and may be precipitated from the extract by addition of excess of alcohol containing hydrochloric acid (*vide infra*). During the extraction, slight de-esterification of the pectin invariably occurs; for this reason, the period of extraction should be reduced as far as possible, compatible with a good yield.

Pectins are also extracted from tissues by prolonged boiling with water, especially if the heating be carried out in an autoclave at 105°–110°. Extraction with very dilute hydrochloric acid (of a strength not exceeding $N/75$), by boiling for periods of 3 hours, removes most of the cell-wall pectin, but does not extract the middle

lamella pectin. Boiling with very weak alkali likewise extracts part of the pectin, but in the form of a salt of pectic acid. The disadvantage of using alkalies as solvents lies in the fact that completely de-esterified products are obtained, which are quite incapable of forming jellies when boiled with sugar.

Organic acids (citric, tartaric, etc.) remove pectins more or less completely on boiling, causing hydrolysis of the protopectin; the former acid especially is used in the manufacture of commercial preparations, but for laboratory use these acids do not offer any special advantage.

(2) **Quantitative Extraction.**—For the purpose of preparing extracts for estimating the various forms of pectin in a tissue, the following methods are recommended.

(i) *Extraction of Soluble (Free) Pectin and Pectinic Acids.*—These substances, being water-soluble, are most satisfactorily extracted from the fresh material (fruit, etc.). The tissue should be treated before extraction in such a way as to disintegrate the cells, and render their contents accessible to the solvent. For this purpose, 50 grm. of the tissue, cut into thin slices and mixed with about 30 c.c. of water, are placed in a freezing mixture for several hours. After being allowed to return to ordinary temperature, the liquid present is pressed out in a small hand-press. More distilled water (30–40 c.c.) is mixed with the residue, and this is again pressed; the process is repeated at least 20 times, the extracts being combined, filtered and made up to a known volume for purposes of estimation. It is found impossible to reach a stage when water extracts no further material, probably on account of the fact that slow hydrolysis of protopectin, or dispersal of colloidal pectic acid, takes place. Carré, however, considers that the above method is satisfactory.

According to Nanji and Norman (*Biochem. J.*, 1928, **22**, 596), the method has two serious disadvantages: (i) the true soluble pectin may not be wholly extracted, while pectic acid or colloidal pectin may become dispersed and pass into the extracts, and (ii) the repeated pressing involves much labour, and becomes impossible when large numbers of estimations have to be carried out. They therefore recommend the following modified technique: The fresh material is finely powdered or thinly sliced, and dried rapidly in a steam-oven, whereby enzyme action is checked. A suitable amount (10–20 grm.) of the dried material, ground and sieved, is digested

with distilled water on a water-bath, the temperature of extraction being kept below 85°. *Extraction is continued for 24 hours, the* water being changed from time to time; the combined filtered extracts are made up to 500 c.c. or 1,000 c.c. for estimation. It seems likely that some hydrolysis of protopectin takes place during this treatment, but the authors apparently consider that the danger is negligible.

(ii) *Extraction of Pectin from Protopectin.*—As has been pointed out already, no satisfactory method has yet been devised for the extraction of protopectin in an unchanged form. The estimation of cell-wall pectic substances is therefore dependent on the use of some method whereby the protopectin will be converted completely into soluble pectin, without further degradation of the latter.

For the purpose of this extraction, dried and powdered material gives the best results. (A method of drying, alternative to that referred to above, and applicable to all plant tissues, is as follows: the thinly sliced material is dropped into 10 times its weight of boiling alcohol, and boiling is continued for 10–20 minutes; all enzymes are instantly destroyed. After the alcohol has been drained off, the material is dried for 2 hours in a steam-oven, after which it is left *in vacuo* for 24 hours over sulphuric acid, and powdered in a coffee-mill. It is found that no loss of pectin occurs on account of solution in the alcohol. In calculating the % dry weight of material, allowance must be made for loss of substance soluble in alcohol.) Extraction of the cell-wall pectin is carried out by means of hydrochloric acid, of a concentration not exceeding $N/75$. Portions of the dried tissue (1–5 grm., according to pectin content) are gently boiled with 100 c.c. of the acid for periods not exceeding 3 hours, under a reflux condenser; periods of hydrolysis exceeding 3 hours, or the use of acid stronger than $N/75$, result in perceptible hydrolysis of the pectins. From 3 to 5 such extractions suffice to remove all the pectin at this stage. The use of a centrifuge may be necessary to obtain a clear extract; the combined extracts are filtered and made up to 500 c.c. or 1,000 c.c. This extract contains, beside the cell-wall pectin, any soluble pectin originally present in the tissue, and allowance must therefore be made for this.

(iii) *Middle Lamella Pectin.*—The residues left after extraction with $M/75$ hydrochloric acid will still contain any pectic substance which was originally present as free pectic acid or as pectate, *i. e.*,

pectins of the middle lamella. These will now be present as insoluble pectic acid. It has been suggested that treatment of the residues with 0.05% sodium hydroxide would remove the whole of this pectic acid, and in fact microscopic examination of the residues after three such extractions shows that no pectin remains. Alternatively, extraction with 0.5% ammonium oxalate solution has been suggested. The material remaining after hydrochloric acid extraction is not, however, usually in a condition suitable for further treatment, having undergone so much disintegration as to be difficult to handle. In determining pectic substances of the middle lamella it is advisable to extract the total pectic substance of the tissue, as described below, and to return as "middle lamella pectin," total pectin less (protopectin plus soluble pectin).

(iv) *Total Pectic Substances.*—The most satisfactory solvent for removing the whole of the pectic substances is 0.5% ammonium oxalate. From 1–5 grm. of the dried and powdered material is treated with 100 c.c. of the solvent, and kept at 85° for 20 hours; the extract is filtered or centrifuged off, and the residue is again treated with ammonium oxalate for 4 hours, whereby the last traces of pectin are removed from the tissue. The extracts are made up to 250 c.c. The estimation of the pectic substances in this and the preceding extracts is carried out by the calcium pectate method described below (p. 529).

Laboratory Preparation of Pectic Substances

(1) **Pectin.**—Preparations of pectin of a fair degree of purity are readily obtained from material such as turnips, oranges, onions, apples etc., which are rich in these substances. Preliminary treatment consists in mincing the material finely, and repeatedly extracting with cold water, in the presence of toluene and chloroform, until further washing removes only a negligible amount of soluble matter. The residue is then boiled with a large amount of 95% alcohol, pressed, and dried in the air, and powdered. From this dry product, the pectin may be extracted by means of any of the solvents referred to above, that most frequently employed being 0.5% ammonium oxalate. Six hours' extraction removes the bulk of the pectin without producing very extensive de-esterification. Addition of 2–3 volumes of 95% alcohol, containing 10 c.c. concentrated hydrochloric acid per litre, to the filtered extract,

precipitates the pectin as a fairly bulky jelly. This is filtered off on a large fluted filter (filtration by suction is useless), well washed with 60–70% alcohol, and may be redissolved in water, and reprecipitated by addition of excess of alcohol. If it is desired to dry the product, this is best done by allowing it to stand in successive portions of alcohol of graded strengths, the pectin being dried afterwards in a current of warm air, and finally to constant weight *in vacuo* over phosphorus pentoxide. The product thus obtained consists of a mixture of the soluble pectins containing, on an average about 9% methoxyl. Some inorganic matter is invariably present, and probably substances other than pectins, derived from the plant tissue (hemicelluloses). For many purposes, such as the preparation of pectic acid, the product is sufficiently pure.

For the preparation of purer samples of pectin, several methods have been suggested, of which the following is recommended by Carré. The extracts are filtered through kieselguhr, and, after boiling, are left to stand for several weeks in flasks plugged with cotton-wool (toluene and chloroform being added to inhibit bacterial action). Suspended impurities settle, carrying down colouring matter. From the clear supernatant liquid, the pectin is precipitated by alcohol, washed and dried in the usual manner.

Purification by electrodialysis has been suggested, but Spencer (*J. Phys. Chem.*, 1930, **34**, 429) considers this unsatisfactory. He states that repeated washing by decantation with acidified alcohol gives the best results, products containing only about 0.1% of ash being obtained. No method is known for removing completely the hemicelluloses, which seem invariably to accompany pectin to a greater or less extent.

(2) **Pectic Acid.**—This substance is generally considered to form the basal unit of all the pectic bodies, and to possess a definite composition. In the examination of plant substances with a view to establishing their identity as pectins, it is therefore usual to prepare pectic acid, and to demonstrate that this substance answers to the composition required by the accepted formula. Pectic acid is readily prepared from pectin, which need not be highly purified, either directly, or better, through the insoluble calcium salt. The method of Schryver and Haynes (*Biochem. J.*, 1916, **10**, 539) consists in treating a 2% solution of pectin with slight excess of caustic soda solution (1 grm. of pectin is equivalent to

roughly 50 c.c. of $N/10$ sodium hydroxide), and allowing the mixture to stand for 12 hours at room temperature. Complete de-esterification is thus effected, and a solution of sodium pectate obtained. Addition of an equal volume of concentrated hydrochloric acid precipitates the free pectic acid as a firm gel. This is filtered off on a large fluted filter, and kneaded in water, in a muslin bag, till free from chloride, being finally dried by means of alcohol.

Alternatively, the alkaline solution of sodium pectate may be neutralised with acetic acid, and excess of calcium chloride solution added. The precipitated calcium pectate is repeatedly boiled with water, decomposed with hot ammonium oxalate solution, and the pectic acid precipitated from the filtered oxalate extract by addition of excess of acidified alcohol. The pectic acid is washed and dried in the usual way, and a preparation of a high degree of purity may be obtained in this manner.

It is often preferable to prepare samples of calcium pectate, for this, like the free acid, is of constant composition, but is more easily handled, since it shows less tendency to become dispersed in water during prolonged boiling. Especially when dealing with small amounts of substance is the use of calcium pectate advised; the exact procedure recommended is that described below (p. 529) for the quantitative estimation.

Commercial Preparation of Pectin

A very large number of patents covering the manufacture of pectin from various sources have been granted, and many proprietary brands of pectin are now obtainable. It is not possible here to give details of the methods of preparation of such products, but the outlines of the methods in use may be mentioned, in order to give some idea of the natures of the products likely to be met with commercially.

The chief sources available for the commercial production of pectin are waste and unsaleable apples, cider residues, small and waste citrus fruits, turnips and other root crops; the fruits used must be sound and not over-ripe, since in such cases the pectins have largely undergone degradation (see p. 538).

In most processes the fruit or pulp is well washed to remove sugars and bitter principles, washing with hot dilute solutions of citric acid being adopted in some cases. Extraction of the pectin

is carried out by heating with water under pressure, or more frequently by heating with weak acids, usually under slight pressure. This results in the liberation of soluble pectin from the insoluble protopectin. The use of 0.05% hydrochloric acid, or 1%–1.5% citric or tartaric acid, has been recommended.

The extract, usually after a preliminary filtration through felt, charcoal or kieselguhr, is either evaporated to a syrup in vacuum pans or is precipitated, with or without previous concentration, by addition of excess of alcohol. The product may be marketed as a syrup, containing up to 15% of solid matter, or as a fine dry powder, having a pectin content of about 70%. Traces of the acid used during extraction may be present, or acid (citric) may be added in small amounts to facilitate jelly formation with sugar. Some preparations also contain considerable amounts of sugar, and are capable of forming jellies merely by dissolving in water, boiling and cooling. In the case of solid preparations, the incorporation of sodium bicarbonate and citric acid has been proposed, in order to promote dispersion of the pectin, which otherwise tends to form stiff clots of jelly on mixing with water.

Characterisation of Pectic Substances

(1) **Qualitative.** (*a*) *Protopectin.*—This substance cannot be extracted from tissues in an unchanged form; for its detection in the plant, it is necessary either to prepare the soluble pectin therefrom, or to rely upon the micro-chemical (staining) tests described below.

(*b*) *Soluble Pectin and Pectinic Acids.*—(i) The members of this group are soluble in water, giving viscous solutions which are strongly dextrorotatory. The addition of 2 volumes of alcohol to the solution precipitates the pectins in the form of a flocculum or gel, redissolving on addition of water.

(ii) Pectins do not reduce Fehling's solution, but on gentle hydrolysis with acids give reducing substances.

(iii) Pectins, on heating with 12% hydrochloric acid and orcinol give the green colour characteristic of pentoses; with 12% hydrochloric acid and naphthoresorcinol, they give a violet colour and fluorescence.

(iv) On heating with nitric acid (sp. gr. 1.15) pectins yield mucic acid.

(v) On allowing a pectin solution to stand for a few minutes with N sodium hydroxide solution, and adding an excess of hydrochloric acid, pectic acid is precipitated as a colourless gel.

(vi) On treatment with lime water, a gelatinous precipitate of calcium pectate, insoluble in dilute acetic acid, is produced.

(vii) The presence of methyl groups is shown by the following test (Sucharipa): 0.5 grm. of pectin is placed in a 150-c.c. distilling flask with 20 c.c. of water, and 5 c.c. of 10% sodium hydroxide added. The flask is corked and allowed to stand for 10 minutes. The soda is then roughly neutralised with sulphuric acid, and the mixture distilled, the distillate being collected in a beaker immersed in a freezing mixture. Methyl alcohol is detected in the distillate by any of the usual tests.

(*c*) *Pectic Acid and Pectates.*—(i) Only the pectates of the alkali metals and ammonium are soluble. Solutions of these pectates are precipitated on addition of alcohol. Calcium pectate is precipitated from such solutions, in the presence of dilute acetic acid, on adding excess of calcium chloride solution.

(ii) Pectic acid and pectates give reactions (ii) to (iv) above; since they contain no ester groups, reactions (v) to (vii) do not apply.

(2) **Micro-chemical Tests.**—The detection of pectic substances in plant tissues by staining is a matter of some difficulty, since pectins are akin to cellulose and occur in association therewith. Moreover, no stain is specific for pectins, and staining reactions must be corroborated by solubility tests. The following tests may be applied:

(i) Methylene blue, Bismarck brown and fuchsin stain lignin, suberin, and pectin, but not pure cellulose; subsequent treatment with glycerin, alcohol, or dilute acids decolorises the pectins, but not lignin or suberin. Crocein stains lignified and suberised tissues, and cellulose, but not pectin. Congo red and azo-blue stain only cellulose. (Mangin.)

(ii) Carré and Horne (*Ann. Bot.*, 1927, **41**, 1) recommend the use of aqueous ruthenium red as being more specific for pectins than any other reagent. The sections are washed with water to remove acids etc., and immediately stained with aqueous ruthenium red (1 in 5,000), which must be freshly prepared. After warming for a few minutes in water, the pectins retain the stain distinctly; other tissues are rendered colourless. Among other substances which

stain with ruthenium red are hemicelluloses, gums, mucilage, fatty acids, gelose and lichenin; these can be distinguished from pectin by the solubility tests described below. (iii) to (vii).

(iii) Pectins are not dissolved from sections by ammoniacal copper solutions.

(iv) On warming the sections with 0.5% ammonium oxalate, the pectins dissolve, but celluloses are unaltered. Prolonged treatment with 20% hydrochloric acid, followed by washing with potassium hydroxide solution, also effects complete removal of the pectins.

(v) Treatment of the sections with 20% alcoholic hydrochloric acid for 24 hours, followed by washing with water, does not remove the pectic substances of the middle lamella; these may be detected by staining subsequently with ruthenium red.

(vi) After the above treatment with acid alcohol, washing with 10% ammonia causes ready separation of the cells. On the other hand, washing with baryta water forms the insoluble barium pectate, and the cells do not part on washing with ammonia.

(vii) Treatment with Schweitzer's solution removes cellulose from the sections, but leaves the pectins, the structure of the section being thus preserved. Staining with ruthenium red stains the sections deeply. Subsequent washing with ammonium oxalate solution destroys the whole of the structure.

(viii) Mehta (*Biochem. J.*, 1925, **19**, 979) describes the following method: The sections are placed in a test-tube containing the extracting reagent, and placed in a boiling water-bath for 6–8 hours, the reagent being replaced every hour. The sections are then washed with hot distilled water, stained for 2 hours, washed with 90% alcohol, dried in absolute alcohol, and mounted in cedar-wood oil. The solvents recommended are: 0.5% ammonium oxalate solution, which removes all pectic substances; 3% hydrochloric acid, which removes protopectin; and 4% sodium hydroxide, which removes free pectic acid, hemicelluloses etc., but not protopectin. The stains used are:—

Malachite green—1 grm. in 300 c.c. of 92% alcohol.
Congo red—0.5% aqueous solution.
Eosin—0.3% aqueous solution.
Iodine green—1% aqueous solution.
Ruthenium red—0.01% aqueous solution.

Full details are given in the original paper.

(3) **Quantitative.**—For the quantitative characterisation of pectic substances, it is advisable to convert the substance into pectic acid or calcium pectate, by the method already given. The yield obtained from a known weight of pectin should be noted. The only case where the use of pectic acid is impossible is in the determination of methoxyl.

(i) *Ash.*—Accepting the ring formula for pectic acid, the formula for calcium pectate is $C_{35}H_{46}O_{33}Ca_2$, containing 7.45% calcium, or 10.42% of ash as calcium oxide.

(ii) *Furfural Yield.*—On distillation with 12.5% hydrochloric acid, the pectins yield furfural, derived partly from the arabinose and partly from the galacturonic acid residues. On the basis of the cyclic formula for pectic acid, the furfural yield for pure pectic acid should be approximately 20.8%, and for calcium pectate, 19.0%. Furfural determinations must be carried out on pectic acid or calcium pectate, since the pectins prepared directly from plant materials invariably contain furfural-yielding bodies other than pectins. The estimation of furfural from pentoses is described in detail in Vol. I, p. 498 *et seq.* Since the furfural yielded by pectins is derived partly from pentose and partly from uronic acid residues, it is usual to express the results as "furfural yield" rather than as "pentose content" of pectin (as in earlier work).

Kullgren and Tyden (*Ing. Vetenskaps. Akad., Stockholm,* 1929, *Handl.* 94) give details of an improved method for estimating furfural, in which the gravimetric determination of the furfural as phloroglucide is replaced by a much more rapid volumetric method. In their method, the distillation is carried out with 13.15% hydrochloric acid (in place of the usual 12%) in the presence of excess (20 grm.) of sodium chloride; this saturated solution of salt in hydrochloric acid gives a constant-boiling mixture. Distillation is carried on for 140 minutes, at the rate of 25 c.c. in 10 minutes, 350 c.c. of distillate being collected. The volume of the distillate is made up to 400 c.c. A portion of this liquid (100 c.c.) is placed in a conical flask, 200 c.c. of 1.58-*N* sodium hydroxide added, the flask is corked and the mixture rapidly cooled to room temperature. 10 c.c. of 2.5% ammonium molybdate solution are added, followed by 25 c.c. of standard bromide-bromate mixture; this contains 1.392 grm. of potassium bromate and 10 grm. of potassium bromide per 100 c.c. (*i. e.*, $N/20$ $KBrO_3$). The flasks are immediately placed

over a white surface, and the moment at which a distinct yellow colour appears is noted; this usually occurs within 30 seconds. From this moment, the solution is allowed to stand for exactly 4 minutes, during which time it has been shown that 1 molecule of *furfural liberates exactly 2 atoms of bromine.* 1 grm. of potassium iodide is then added, the mixture well shaken, and allowed to stand for a further 10 minutes, when the liberated iodine is titrated in the usual manner with $N/20$ thiosulphate, in the presence of starch. If, for the whole 400 c.c. of distillate, the amount of $N/20$ bromate is n c.c., the amount of furfural contained in the distillate is $n \times 0.0024$ grm.; for calculating the amount of furfural given by the sample of pectin, this value must be corrected to allow for the 3.1% of furfural which does not pass over into the distillate.

If hexoses are present in the sample, it is recommended by Kullgren and Tyden that 100 c.c. of the distillate be redistilled in the presence of 20 grm. of sodium chloride, and 100 c.c. of this second distillate collected, 25 c.c. of fresh acid being added to the mixture every 10 minutes during the distillation, as before. Titration of this second distillate is carried out in the usual manner, allowance again being made for the fact that only 96.9% of the furfural is capable of distillation. In the case of pectic substances, in which the amount of hexose is comparatively small, this second distillation seems unnecessary.

The original paper gives further factors connecting titration values with arabinose, xylose, rhamnose, and pentoses in general.

(iii) *Carbon Dioxide Yield (from Uronic Anhydride Residues)*.—The presence of uronic anhydride residues as fundamental units in the pectin molecule was demonstrated by Ehrlich (1917), who isolated galacturonic acid from among the hydrolysis products. The estimation of the uronic anhydride content was rendered simpler by the observation of Nanji, Paton and Ling (*loc. cit.*), that these substances yield carbon dioxide quantitatively on distillation with 12.5% hydrochloric acid, the yield from the acids being 22%, and from the anhydrides 25%, of the weight taken. The method described by Nanji, Paton and Ling is as follows.

A suitable weight of pectin (0.3–0.5 grm.) is placed in a wide-necked round flask of 150 c.c. capacity, fitted with a reflux condenser, and placed in a bath of glycerine. 60 c.c. of 12.5% hydrochloric acid are added, and a current of air, carefully freed from carbon

dioxide by passing through a series of potash or soda-lime tubes, is led in at a speed of about 3 bubbles per second. A tube from the upper end of the condenser leads to a small bubbler, containing a paste of silver sulphate, and thence to a series of 3 gas towers, each containing 100 c.c. of $N/20$ barium hydroxide solution. The exit from the last tower is protected by a soda-lime tube. Glass taps are provided between the towers, so that any tower may be cut out of the series, and disconnected; the baryta solution is measured into the towers from an automatic pipette. After connecting the towers, the glycerine bath is slowly heated until the acid in the flask boils gently; heating is continued for 3 hours, by which time the evolution of carbon dioxide is complete. If the air-current has not been too rapid, only the first two towers contain any deposit of barium carbonate. The towers are closed, disconnected, and the barium carbonate therein allowed to settle. 25 c.c. portions are withdrawn from each, and titrated with $N/20$ hydrochloric acid, using phenolphthalein as indicator. A "blank" experiment must be carried out, and the titrations corrected thereby. The theoretical yield of carbon dioxide from calcium pectate amounts to 16.38%, equivalent to a uronic anhydride content of 65.52%; for pectic acid, the figures are respectively 17.64% and 70.56%.

A micro-method for the determination of uronic anhydride has been described by the writer (*Analyst*, 1932, **57**, 220), and offers certain advantages. It permits of determinations being carried out with 5–10 mg. of the material, and at the same time the apparatus required is less cumbersome. The results show the same order of accuracy.

A method for the simultaneous determination of furfural and carbon dioxide has been proposed by Bowman and McKinnis (*J. Amer. Chem. Soc.*, 1930, **52**, 1209). Briefly, the method consists in distilling a sample (0.3 grm. of pectin) with 12% hydrochloric acid in a current of CO_2-free air and steam. The distillate is collected in a flask cooled by a freezing mixture, whereby the furfural is completely condensed. An outlet from this flask leads the issuing gas over hot moist zinc powder ("mossy zinc"), in order to retain any hydrochloric acid, and thence into 2 gas towers containing standard baryta solution. The distillation is continued for 3½–4 hours; furfural is estimated by the phloro-glucide method, and carbon dioxide by titration of the excess baryta. Full working

details and a diagram of the apparatus are given in the paper quoted.

(iv) *Methoxyl Content.*—It has been shown by a number of workers that the ability of pectins to form jellies with cane sugar, the property on which the main commercial value of pectins depends, is a function of the methoxyl content of the pectin. The measurement of the methoxyl content thus becomes a matter of considerable importance. The most suitable method is that of Zeisel, in one of its modifications. A sample of solid pectin should be used, in order to prevent dilution of the hydriodic acid; the sample should be ground to a fine powder, and dried for some hours at 100°. The method due to Hewitt and Jones (*J. Chem. Soc.*, 1919, **115**, 193), in which the gravimetric determination of the silver iodide is replaced by a much more rapid volumetric method, is probably most convenient. The apparatus consists of a distilling flask (150–200 c.c. capacity) with a neck 12″ long, the side-tube being close to the top of the neck. The side-tube is bent over and passes into a test-tube containing 10 c.c. of pyridine; a second test-tube connected in series, also containing 10 c.c. of pyridine, completes the absorption apparatus. Throughout the determination a slow stream of carbon dioxide is passed through the apparatus. 20 c.c. of hydriodic acid (sp. gr. 1.7; b. p., 126°) are placed in the flask, which is then immersed in a glycerin bath heated to 130°, while carbon dioxide is passed. This process serves to remove volatile impurities from the acid, and is continued until the issuing gas no longer produces a turbidity in silver nitrate solution. The apparatus is allowed to cool somewhat, the absorption tubes attached, and the sample of pectin (0.3–0.5 grm., weighed out into a small glass capsule) is dropped into the flask. The glycerin bath is heated until the acid boils gently. After 1 hour, the contents of the two test-tubes are washed into a graduated flask, and the volume is made up to 100 c.c. 25 c.c. of this liquid are placed in a stoppered bottle, 70 c.c. of water added, and then, in order, 25 c.c. of $N/10$ silver nitrate and 30 c.c. of 10N nitric acid. The bottle is stoppered and well shaken for 5 minutes, after which the liquid is titrated with $N/10$ thiocyanate solution, in the presence of ferric alum indicator, until a permanent orange colour appears. The results obtained by this method are usually slightly low.

If a micro-balance is available, the use of the micro-Zeisel method described by Pregl (*Organic Micro-analysis*, 1924, p. 150) is advised.

This is a gravimetric method in which the silver iodide formed by passing the vapour containing methyl iodide into alcoholic silver nitrate solution, is collected on a special micro-filter and weighed. The accuracy attainable is greater than that of the volumetric method described above, and the whole determination can be carried out (including all weighings) in 60–90 minutes.

(v) *Mucic Acid Yield.*—The galactose and galacturonic acid residues present in the pectin molecule give rise to mucic acid on oxidation with nitric acid of sp. gr. 1.15. The determination of mucic acid yield is not usually necessary, but may in some cases be advisable in order to establish definitely the identity of the pectic acid. The method of Tollens is described fully in Vol. I, p. 472. Approximately 55%–60% yields of mucic acid are obtained from pectic acid, but the amount varies somewhat considerably; the theoretical value is approximately 65%.

The following table summarises the various constants for pectic acid and its derivatives, on the basis of the Nanji, Paton and Ling formula.

	Pectic acid	Calcium pectate	Soluble pectins			
			Tetra- methyl	*Tri-* methyl	*Di-* methyl	*Mono-* methyl
Calcium content, %	......	7.45	......	......	......	......
Furfural yield, %	20.8	19.2	19.5	to	20.7	......
Carbon dioxide yield, %	17.64	16.38	16.9	to	17.4	......
Uronic anhydride content, %	70.56	65.52	67.7	to	69.6	......
Mucic acid yield, %	65	60	60	to	65	......
Methoxyl content, %	......	......	11.76	8.94	6.04	3.06

Estimation of Pectic Substances.—The quantitative determination of pectic substances is a matter of some considerable importance from a commercial standpoint, since a determination of the amount of pectin in a commercial preparation serves as a guide to its quality. Pectin determinations are also of value in assessing the storage properties of fruits, and their suitability as sources of commercial pectin.

The following methods are available for determining pectins in commercial products, or on extracts of plant material prepared as described above (p. 518). Of these, the methods in section (i), involving direct precipitation with alcohol, are only very approximate, since other substances apart from pectin will appear in the precipitates. For accurate determinations, methods based on the precipitation of calcium pectate are recommended.

(i) *Estimation by Precipitation with Alcohol.*—Although not specifically given as a method for estimation of pectin in fruit juices, the A. O. A. C. method for "alcohol precipitate" approximates to a rough estimation of pectic substances (*A. O. A. C. Methods of Analysis*, 1931, p. 268). 60 grm. of fruit syrup or jelly are dissolved in water, the volume is made up to 300 c.c., and the liquid is thoroughly mixed. 100 c.c. are taken and evaporated to 20 c.c.; 200 c.c. of alcohol (96% by volume) are added slowly with constant stirring, and the mixture is allowed to stand overnight. The mixture is filtered, and the precipitate is washed on the filter with 86% alcohol. It is then washed from the filter with hot water into a platinum dish, evaporated to dryness and weighed. The residue is ignited, and the ash weighed, the difference between the weight of the dry residue and that of its ash being designated "alcohol precipitate." The amount of ash should not exceed 5% of the total dry precipitate, and should be chiefly lime. If the amount exceeds 5%, the water soluble portion of the ash is titrated with $N/10$ acid, as any potassium bitartrate brought down by the alcohol can thus be estimated.

Wichmann (*J. A. O. A. C.*, 1922, **6**, 35; 1923, **7**, 107; 1924, **8**, 123) recommends the following further treatment of the alcohol precipitate: after washing with 86% alcohol, it is redissolved in hot water, the solution evaporated to 15 c.c., and finally treated with 200 c.c. of alcohol (96%) containing 5 c.c. of 10% hydrochloric acid. The precipitate is washed, dried, weighed and ignited as in the original A. O. A. C. method.

These methods give, at best, only a rough approximation, and are not recommended for accurate work.

(ii) *Estimation as Calcium Pectate.*—The estimation of pectic substances as calcium pectate, a method first introduced by Carré and Haynes (*Biochem. J.*, 1922, **16**, 60), forms the only satisfactory process for the accurate determination of these substances. Calcium

pectate is insoluble, even in extreme dilutions, and is a substance of definite composition into which all pectins are readily converted. Its insolubility in dilute acetic acid allows the removal of the calcium salts of all organic acids commonly met with in plant products, except oxalic acid; a modification of the method, given below, is available when oxalates are present.

A quantity of the pectin solution, which will yield approximately 0.02–0.05 grm. of calcium pectate, is roughly neutralised and made up to 300 c.c. 100 c.c. of $N/10$ sodium hydroxide are added, to de-esterify the pectins and pectinic acids, and to convert them into sodium pectate. The process takes place extremely rapidly, and is complete under these conditions in a few minutes. It is however advisable to allow the mixture to stand for 1 hour (Carré recommends overnight), since this results in a physical change whereby the consistence of the calcium pectate gel subsequently precipitated is altered, and the gel is rendered more readily filterable. 50 c.c. of $N/1$ acetic acid are then added, and, after standing 5 minutes, 50 c.c. of $M/1$ (11%) calcium chloride solution. A gelatinous precipitate of calcium pectate forms immediately. After standing for 1 hour, the mixture is carefully boiled for 1–2 minutes, when the voluminous precipitate is reduced to a small and compact, but still gelatinous form. The liquid is liable to froth considerably at the commencement of boiling, and should therefore be watched carefully. The precipitate is filtered off on a smooth-surfaced paper, and is washed on the paper with about 100 c.c. of water. It is then washed back into the beaker with 250 c.c. of water, and the mixture again boiled for 1 minute. The filtering, washing and boiling are repeated until the filtrate is free from chloride. This result can only be obtained by actually boiling the suspended calcium pectate; three boilings are found to be adequate in most cases. The precipitate is finally filtered on to a tared Gooch or alundum crucible, dried at 100° for 12 hours, and weighed.

The method can be used for very dilute pectin solutions (0.01%), such as are unprecipitated by alcohol. Carré recommends that in such cases the amount of calcium chloride used should be decreased.

The criticism has been made that repeated boiling tends to redissolve or redisperse the precipitate; if the standard conditions are adhered to, this is not the case. In the experience of the writer, however, it is essential, in order to obtain accurate and comparable

results, to standardise the procedure rigidly, both as regards quantities of reagents used, length of time allowed for the various stages, time of boiling, and quantity of water used for washing at each stage. If these precautions are observed, the results are entirely satisfactory.

(iia) *Modified Calcium Pectate Method.*—As pointed out above, the calcium pectate method is liable to error if the extracts under examination contain acids such as oxalic acid, whose calcium salts are insoluble in dilute acetic acid. In these cases, however, satisfactory results may be obtained by carrying out a preliminary precipitation with alcohol in the presence of a little hydrochloric acid, since it has been shown (Emmett and Carré, *Biochem. J.*, 1926, **20**, 564) that pectins are quite insoluble in acidified alcohol: The pectin solution is precipitated with 4 times its volume of absolute alcohol containing 1.5 c.c. of concentrated hydrochloric acid per 100 c.c. (*i. e.*, $N/8$ alcoholic HCl, the final mixture after addition to the pectin solution having a normality of $N/10$). After standing overnight, the gelatinous precipitate is filtered off on a rapid paper, washed off the paper with 90% alcohol containing a trace of hydrochloric acid, collected again on the same paper, and washed once with 95% alcohol. It is redissolved in warm water, and the solution, diluted to 300 c.c., is used for determination of calcium pectate, as described above.

Norman (*Biochem. J.*, 1928, **22**, 596) has pointed out that, if a plant tissue contains insoluble pectate (*e. g.*, calcium pectate in middle lamella) this will pass into an oxalate extract and be precipitated by acid alcohol as free pectic acid. The latter, being insoluble in water, will be lost on redissolving the precipitate. For this reason he suggests that the alcohol precipitate should be redissolved in weak ammonium hydroxide, when any free pectic acid will pass into solution as ammonium pectate.

The modified method is especially suitable when dealing with the ammonium oxalate extract used for estimation of "total pectin" in plant tissues. The oxalate extract may be precipitated directly with acid alcohol, but it is frequently advisable to evaporate the extract to one-fourth or one-fifth of its volume before precipitation.

Extracts made with $N/75$ hydrochloric acid (estimation of "protopectin") can usually be estimated as calcium pectate without preliminary precipitation with alcohol. In the case of some tissues,

notably leaves, where strong organic acids are likely to occur, it is best to use the modified method. In this case, the hydrochloric acid extract should be nearly neutralised with dilute sodium hydroxide, and evaporated to one-fifth its volume before the addition of alcohol.

It is obvious that the calcium pectate method does not give an estimate of the individual pectic substances in the tissue, but only an estimate in terms of calcium pectate. The substances actually present—protopectins of varying composition, soluble pectins, etc.—cannot be estimated absolutely, since no means are known of isolating these compounds in an unchanged state. The calcium pectate method does, however, give a basis for the comparison of the amount of pectins present in different materials, and of the amount of the different types of pectin in one tissue. The results are best expressed as "calcium pectate number," *i. e.*, grm. of calcium pectate yielded by 100 grm. of dry tissue or sample.

(iii) *Other Methods.*—Various of other methods have from time to time been suggested for the estimation of pectins. von Fellenberg suggested the use of methoxyl determinations, assuming that all the pectin present was in the fully methylated form (11.76% methoxyl). It is obvious that this is not permissible, since not only is a portion of the pectin known to be only partly in the esterified form, but other substances may be present (*e. g.*, lignins) which contain methyl groups. The same objection applies to the suggestion of Nanji, Paton and Ling that uronic anhydride content should be taken as a basis for estimation; hemicelluloses, gums etc., which are nearly always associated with pectins, also yield carbon dioxide on treatment with hydrochloric acid.

(iv) *Methods for assessing the value of pectins* for jelly manufacture are given in a later section (p. 535).

The Formation of Pectin-sugar Jellies.—The chief commercial importance of the pectins lies, as has been pointed out, in their use for the preparation of fruit jams and jellies. A knowledge of the conditions governing the formation of such jellies is, therefore, desirable, in order that some measure of control may be exercised in the manufacture of the products.

The ability of pectin (*i. e.*, fruit extracts), when boiled with sugar, to form jellies which readily "set" has been recognised for a considerable time, and was at first ascribed to the conversion of

pectin to pectic acid, which was known to be produced in the form of a gel by the action of certain plant enzymes. That such is not the case is shown by the work of Sucharipa, who was able to recover unchanged soluble pectin from the jellies by prolonged washing with alcohol, and further was able to prove that the formation of such jellies depended on the presence of soluble (esterified) pectin. It follows, therefore that although a fruit may be rich in total pectic substances, it may be valueless as a source of pectin for jelly making, unless the pectin can be isolated in a soluble, esterified form. The determination of the distribution of the different types of pectin in the fruit thus becomes of great importance. Further, the presence of acid is essential for the formation of jellies, and the addition of acid is therefore necessary in the case of fruits naturally deficient in these substances. One may classify fruits in the following manner, on the basis of their content of pectin and/or acid, to give an idea of their value in the manufacture of jams and jellies:

(a) Fruits rich in soluble pectin or in protopectin and acid; capable of forming jellies on boiling with sucrose alone; *e. g.*, sound apples, crab apples, currants, loganberries, plums, oranges, and quince.

(b) Fruits rich in available pectin, but deficient in acid; require the addition of acid to liberate soluble pectin from protopectin, and to form jellies with sugar; *e. g.*, pears, peaches, melons, sweet apples.

(c) Fruits deficient in both pectin and acid; require the addition of pectin preparations, or other fruit juices, to form jellies; *e. g.*, strawberries, cherries, rhubarb, pineapple; any too fully ripe or unsound fruit.

The conditions governing the formation of pectin-sugar jellies may be summarised as follows:

(i) *Pectin.*—As stated above, the ability to form jellies with sugar is peculiar to the soluble pectins (methyl esters of pectic acid). Pectins containing less than 7.3% methoxyl have been shown to be incapable of forming such jellies. The majority of commercial pectins contain about 8–9% methoxyl; since they also contain non-pectin impurities, a correction of the methoxyl value (based on their calcium pectate yield) gives a figure of about 11% for the methoxyl content of the true pectin present.

Other conditions being suitable, a concentration of pectin in the final product of about 0.7% gives a satisfactory jelly. Concentrations below 0.3% do not form jellies; concentrations above 0.9% result in the formation of jellies which solidify while still hot.

(ii) *Sugar.*—The amount of sugar used exercises a controlling influence on the nature of the jelly formed. Excess of sugar results in the production of a "soft" jelly; deficiency of sugar gives a "stringy" jelly, and may also be insufficient to prevent the growth of moulds. Cruess and McNair (*Ind. Eng. Chem.*, 1916, **8**, 417) concluded that, in the case of fruits rich in pectin and acid, the fruit juice—sugar mixture gave the best jelly when reduced by boiling to a concentration of 65° Brix. They suggest the following formula for calculating the amount of sugar to be added to the juice:—

$$S = (65 - a) \times \frac{V}{35},$$

where S = grm. sugar necessary; a = °Brix of original juice; V = volume of juice. With the calculated amounts of materials, the mixture need be boiled for only a few minutes to give a firm jelly. In all cases a good quality cane sugar or refined beet sugar should be used. The question has been investigated more fully by Luers and Lochmüller (*Kolloid Z.*, 1927, **42**, 154), who examined the strengths of jellies formed by various amounts of sugar and pectin at a definite acidity, by means of a device known as the pectinometer, which is described below. They found that a jelly of a given strength could be produced by varying proportions of pectin and sugar (*i. e.*, a deficiency of sugar could be rectified, within limits, by addition of an excess of pectin), but that for all the concentrations of pectin investigated, (0.5%–0.9%), the optimum concentration of sugar lay in the region of 60%. The jelly strength for a given concentration of pectin increased rapidly with increase of sugar, up to 60%, but then increased only slowly. They recommend therefore that sugar be added to give a concentration of 60% in the final jelly. (This is in close agreement with the results of Cruess and McNair.)

(iii) *Acidity.*—The presence of acid is essential for the formation of pectin-sugar jellies. The optimum acidity lies at *p*H 3.0, the

maximum on a pH-jelly strength curve being very sharp. When the fruit is deficient in acid, the addition of 0.2–0.3 grm. of citric or tartaric acid per 100 c.c. of the product gives a satisfactory result. Many pectin preparations (*e. g.* those from citrus fruits) contain acid, and the use of additional acid is unnecessary.

(iv) *Time of Boiling.*—Prolonged boiling rapidly destroys the jelly-forming power of pectin, probably owing to hydrolysis of the ester groups. The mixture should therefore be boiled rapidly, the period being reduced to about 15 minutes if possible. In less than 90 minutes the ability to form jellies is entirely lost.

Methods of Assessing the Value of Pectin Preparations for Jelly-making

(i) **By Means of the Pectinometer.**—Since the formation of stable jellies by pectin depends not only on the total amount of pectic substance present, but also upon its degree of esterification, a simple determination of the calcium pectate value of a commercial preparation is not a sufficient indication of its value from this point of view. Methods are therefore necessary for the rapid assessing of the value of such preparations in jelly-making.

In one method, experimental jellies are actually prepared, and their strength estimated by means of the pectinometer. This apparatus is illustrated in the accompanying figure; it is made by F. and M. Lautenschläger, of Munich. The apparatus consists of a German-silver beaker, 2.75″ in diameter, and 2.75″ high, the sides of which are deeply ribbed, in order to retain the jelly. In the centre of the base of the beaker is a short socket, into which fits a metal rod which is kept vertical by passing through a metal cover or guard at the top of the beaker. The metal rod carries, near its lower end, a device for "tearing" the jelly, consisting of a circle of metal enclosing a cross, as shown in the smaller figure. A fine chain, attached to the rod by means of a hook and loop, passes over two pulleys to a balance pan. The pulley above this pan carries a pointer, moving over a scale.

In testing a sample of pectin, a sugar-acid-pectin jelly is made, under standard conditions, as follows. If the preparation is a syrup or solution, its total calcium pectate value is determined, and an amount of the syrup taken such that the final jelly shall

contain 0.7% pectin (as calcium pectate). This amount is given by the formula

$$X = \frac{0.7 \times 100}{p},$$

where p = calcium pectate value. If X is found less than 15 c.c., water is added to make up this volume; if greater than 15 c.c., the preparation is modified as described below. In the case of solid preparations, 1.5 grm. are dissolved in 13.5 c.c. of water, the calcium pectate number determined, and X calculated as above. 100 c.c. of sucrose solution, of sp. gr. 1.228, are added, and, if necessary, 0.2 grm. of tartaric acid. (The sugar solution is made by dissolving 60 grm. of sucrose in 100 c.c. water.) The mixture is distilled from a 500-c.c. round flask, heated in an oil-bath at 150°. The flask has a short neck (2" long), and the distillate is collected in a measuring cylinder. Distillation is carried out so that 38 c.c. of distillate pass over in 15 minutes, *i. e.*, the mixture remaining in the flask amounts to 100 grm. (15 grm. pectic solution + 122.8 grm. sucrose solution + 0.2 grm. acid = 138 grm.; less 38 c.c. water = 100 grm.). If the pectic solution amounted to over 15 c.c., distillation is continued at the same rate until the excess of water has passed over, leaving the standard 100 grm. of mixture in the flask. The hot mass is at once transferred to the pectinometer beaker, and the metal rod is inserted into the socket. The whole is left for several hours in a cool place, the beaker being immersed to within an inch of its rim in running water. When the jelly has set, the beaker is placed upon the stand of the pectinometer, the chain attached to the rod, and fine lead shot is poured into the balance pan. The pointer on the scale moves at first to the stretch of the chain; it then remains at rest until the addition of sufficient shot causes the jelly to begin to tear. At this point, addition of shot is stopped, and in a few seconds the jelly parts completely. The weight of shot added between that required to stretch the chain and that required to tear the jelly is taken as the "jelly-strength" (in grm.). *It is essential that fine shot be used, and that the* addition thereof should be made at a steady rate, the time taken for the addition of the shot being the same in a series of determinations.

The following example will make clear the method of estimation.

Calcium pectate value of syrup = 2.64%
Amount of syrup taken = 0.7 × 100/2.64 = 26.5 c.c.

Amount of sugar added = 100 c.c. of solution, sp. gr. 1.228
Amount of acid added = 0.2 grm.
Amount of distillate = 38 + (26.5 − 15) = 49.5 c.c.
Rupture of jelly required 321 grm.
Hence jelly strength = 321. *p*H of jelly = 3.05.

Using this method, a jelly whose strength lies between 250 and 350 is considered satisfactory.

(ii) **Chemical Method.**—A purely chemical method of assessing the jelly-making value of pectin preparations, which, it is claimed, gives results parallel to those obtained by the pectinometer, is described by Griebel and Weiss (*Z. Unters. Lebensm.*, 1929, **58**, 189). The method consists in estimating (*a*) the amount of alcohol precipitate given by the sample, under standard conditions; (*b*) the amount of calcium pectate (%) given by the alcohol precipitate; (*c*) the methoxyl content of the sample. The methoxyl value is then recalculated, on the basis of "pure pectin in sample," by corrections obtained from (*a*) and (*b*). The details are as follows:

(*a*) 15–30 grm. of the syrup, diluted if necessary, are filtered with the aid of a pump. The filtrate is precipitated with 10 volumes of 96% alcohol, containing 3 drops of concentrated hydrochloric acid per 100 c.c.; the mixture is well stirred, and allowed to stand overnight. The clear alcoholic solution is decanted, and the residue is transferred to a Buchner filter, if necessary with 96% alcohol, and subjected to strong suction, while being pressed with a flattened glass rod. It is washed twice with dry ether, re-dissolved in 20–30 c.c. of water, warmed to 50°, and again precipitated with alcohol, as before. The precipitate is well washed with alcohol, and then with dry ether, until it is obtained as a fine powder, not sticking together when pressed between the fingers. It is transferred to a weighed flask, warmed to 50°, and dried *in vacuo* to a constant weight.

(*b*) Calcium pectate is determined in a sample of the alcohol precipitate, by the method given above (p. 529).

(*c*) Methoxyl is determined in the original sample by the Zeisel or micro-Zeisel method (p. 527). The methoxyl value obtained here is corrected first by the factor obtained for the amount of alcohol precipitate, and then for the calcium pectate factor of this precipitate.

If the corrected methoxyl value lies above 12%, the preparation will give a strong jelly when boiled with 60% sucrose at *p*H 3.0. The following table, quoted from Griebel and Weiss's paper, shows

the parallel between the corrected methoxyl values and the jelly strengths determined by pectinometer.

Methoxyl value, (corr.)	Jelly strength	*p*H of jelly
10.55	128	2.9
10.75	190	2.9
11.56	255	3.1
12.46	267	3.1
12.65	321	3.05

Further Industrial Significance of Pectic Substances

Apart from their use in the manufacture of jams and jellies, the pectins are involved in many other problems of industrial significance, some of which are mentioned briefly below. The examples quoted by no means exhaust the list.

(1) **Storage of Fruit.**—It has been mentioned above that, for the preparation of commercial pectins, it is essential that sound and not over-ripe fruit be used. This follows from the fact that the pectic substances in the fruit undergo marked changes, both in amount and in nature, during ripening and senescence of the fruit. The problem has been studied mainly in the case of the apple (see numerous papers by Carré and co-workers, *Biochem. J.*, and *Ann. Bot.*, 1922–27), and the conclusions may be summarised thus: In the very young fruit, the amount of free pectin is extremely low, but protopectin in the cell-wall is relatively abundant. During growth, the soluble pectin does not increase greatly, but protopectin steadily accumulates. When ripening approaches, the soluble pectin increases, while the amount of protopectin, though always much greater than that of the soluble pectin, remains constant, or slowly decreases; the change probably involves hydrolysis of the protopectin by the acids (or possibly enzymes also) in the fruit juice. During storage, if the fruit was picked when barely ripe, little change takes place for some weeks, but, after this period, a fairly rapid breakdown of all pectic substance occurs. This breakdown coincides, as would be anticipated, with softening of the fruit, *i. e.*, breakdown of the cell-wall. Different varieties of fruit exhibit different "keeping" properties, and, in general, "keeping" property

of the fruit depends on the amount of protopectin present at maturity, and on the amount of free acid (and pectic enzymes) present. Thus it should be possible to estimate the suitability of a given kind of fruit for storage, by a study of the distribution of the pectins therein, and also to control the storage by periodical pectin estimations.

It has been suggested by Carré and Horne (*Ann. Bot.*, 1927, **41**, 1) that the course of pectin breakdown during storage can be followed perfectly satisfactorily by micro-chemical tests only, and hence that no elaborate determinations of pectin distribution are necessary. Senescence is detected by a marked thinning of the cell-walls, and by a decrease in the amount of middle lamella substance.

A parallel subject is the study of certain diseases in plants, both in fruits and in root vegetables (*e. g.*, "soft-rot" of stored carrots, "wet-rot" of potatoes in clamps, etc.). Here, again, in many cases, the disease involves the breakdown of the pectic material by pectic enzymes, secreted by bacteria or fungi, with consequent separation of the cells. These conditions may similarly be detected by the micro-chemical tests given above.

(2) **Retting of Flax.**—The process known as the "retting" of flax and other fibre-yielding plant materials consists essentially in the solution, by pectic enzymes, of the pectic substances which bind the fibres together. The fibres, which are required, are found in "fibre-bundles," lying near the cortex of the flax stem. The whole of the tissue is marked by a high pectin content, the fibres themselves being of a pectic nature, and bound together by other pectins, differing in solubility and methoxyl content. Thus the organisms which are active in causing retting are also able to attack the fibres themselves, as well as the binding material. If retting is allowed to proceed too far, therefore, some degradation of the individual fibres occurs, with consequent loss of quality in the final product. The "technical end-point" of the ret (*i. e.*, the stage where solution of the pectic binding material is complete, but the fibre-bundles are still intact) may be determined by following the course of retting by means of micro-chemical investigation of the pectic substances in the tissue from time to time, the end point being reached when these tests show that the pectins of the fibres themselves are being dissolved.

Pectic Enzymes.—Frequent reference has been made to the pectic enzymes, and no account of the pectins would be complete without some description of the enzymes associated therewith, since many of the changes which pectins undergo in the tissues, both normal and diseased, are brought about by enzyme action. The relations between the pectic substances and the enzymes are made clear by the following table.

Standard nomenclature	Nomenclature commonly used	Substrate	Products of action
Protopectinase	....	*Protopectin*	*Pectins*
Pectinase	Pectase	Pectin and pectinic acids	Pectic acid
Pectase	Pectinase	Pectic acid and pectates	Sugars, etc.

(The names of the enzymes given in column I, and used below, are those derived by adding the termination "-ase" to the name of the substrate; those in column II are, however, those more frequently met with in the literature. The confusion is unfortunate.)

Little is known of *protopectinase,* and its existence has been questioned. From the industrial point of view it is unimportant, since the reactions ascribed to it can be carried out readily by means of weak acids.

Pectinases are of widespread occurrence, in leaves, fruits, roots etc., the extracts of some plant tissues causing the conversion of pectin to pectic acid in a remarkably short time. They are only of interest commercially in so far as their presence in a plant extract may lead to rapid de-esterification of the pectin, with consequent loss of jelly-forming power. In the usual manufacturing methods, however, the enzymes are destroyed by the preliminary boiling of the tissue. The juice of the fruit should be removed from contact with the cell-wall material as soon as possible after mincing or crushing, since otherwise pectinase action may be appreciable.

Pectases.—From the commercial standpoint, the most important group of pectic enzymes are the pectases, which are responsible for many of the changes met with in manufacturing processes, storage of fruit and vegetables, etc. Enzymes of this type are secreted

by many bacteria and fungi, having been isolated from cultures of these organisms; their action results in the complete hydrolysis of the pectins to sugars and their degradation products. These reactions are involved in plant diseases and in fermentations such as fibre-retting; similar reactions are also involved in biological decomposition of plant material in the production of artificial manures.

Methods are desirable for the comparison of the activities of pectin-splitting enzymes, whether in sterile media (preparations obtained from fungi etc., by alcohol precipitation) or produced *in situ* directly by micro-organisms, in order to permit of a more effective control of industrial pectin fermentations. Little has been done in this direction, however. A method for comparing the activity of samples of pectase has been suggested by Norman (*Ann. Bot.*, 1929, **43**, 233) and has been used by the writer with success. The method involves the decomposition of pectin solutions by enzyme preparations, or by cultures of micro-organisms, the course of the reaction being followed by periodic determinations of unchanged pectin by the calcium pectate method.

The medium used has the following composition:—

Peptone	10 grm
Potassium phosphate	2 grm.
Magnesium sulphate	1 grm.
Pectin, approx.	5 grm.
Water, to	1,000 c.c.

The pectin is first dissolved in about 250 c.c. of water, and the solution filtered; the peptone is dissolved in a further portion of water, followed by the salts. The two solutions are then mixed, diluted to 1,000 c.c., and 20 grm. of precipitated, washed calcium carbonate stirred in, using a mechanical stirrer or shaker. The medium is placed in test tubes, (10 c.c. being measured into each tube) which are plugged with cotton-wool, and sterilised by steaming on 3 successive days for periods of 20 minutes. The sterile tubes are inoculated with the organism, or with a sterile enzyme solution, and incubated at 30°. Calcium pectate determinations are carried out before inoculation, and at intervals every few hours, duplicate determinations always being made. If each tube contains 10 c.c. of the medium, the amount of pectin initially is about 0.05 grm. per tube; the calcium pectate determinations can therefore be carried

out using the total contents of each tube, without further measuring etc. Controls with sterile (uninoculated) tubes or with tubes containing the boiled enzyme, should also be set up. The rate at which the calcium pectate content of the 10 c.c. samples falls is a direct measure of the activity of the enzyme or the organism.

Substances Allied to Pectins; Hemicelluloses.—In plant tissues there are always found, in close association with the pectins, a variety of other carbohydrates, notably hemicelluloses and lignins; these bodies have certain properties in common with the pectins, and have been regarded by some as derived from them. Since the hemicelluloses resemble the pectins to some extent in their solubilities, commercial preparations of pectin are liable to contain them as impurities. The most carefully prepared pectin does not yield 100% calcium pectate, and in fact the figure rarely exceeds 90%, the balance being probably accounted for by inorganic matter and hemicelluloses.

The term "hemicellulose" covers a rather wide variety of substances; some of these appear to be true polysaccharides, yielding on hydrolysis sugars such as glucose, galactose, mannose, xylose and arabinose in varying proportions; others more closely resemble pectin, in containing uronic acid residues. For these, the name "polyuronide" has been suggested. The amount of uronic acid is variable, but is usually much lower than that in the true pectins—the uronic anhydride content of most hemicelluloses lies between 10% and 40%, compared with the 70% present in pectic acid and its derivatives. Some of the hemicelluloses are soluble in hot water, but all are extracted from the tissues by 4% sodium hydroxide. As stated above, they are almost invariably found in association with pectins in plant tissues; in general, non-lignified tissues contain much pectin and little hemicellulose, whilst in lignified tissues the hemicelluloses are in excess.

At present no commercial use seems to have been found for these compounds; their use in the textile industry for "dressing" fabrics has, however, been suggested. Since they are readily available in very large amounts, (*e. g.*, from sawdust, etc.) it is possible that they may in due course become of commercial importance.

(a) *Extraction and Preparation.*—The hemicelluloses are extracted from plant material, usually after this has been thoroughly washed with water, alcohol and hot ammonium oxalate, by prolonged

treatment with 4% sodium hydroxide in the cold. On neutralisation of the extract with acetic acid, part of the hemicellulose may be precipitated, but the whole is only obtained by further addition of excess of alcohol or acetone. Further fractionation of the precipitate has been reported by Norris and Preece (*Biochem. J.*, 1930, **24**, 59), based on the fact that the hemicelluloses form indefinite copper compounds, some of which are soluble in water, and may be fractionally precipitated by acetone.

Separation from the pectins usually depends upon the removal of the latter from the tissue by means of ammonium oxalate; some hemicellulose invariably accompanies the pectin so removed, and can only be recovered by precipitation of the pectin as calcium pectate by lime water, when the hemicelluloses remain in solution.

A method for the purification of the hemicelluloses is that of Baker and Pope (*J. Chem. Soc.*, 1900, **77**, 696).

(b) *Properties.*—The hemicelluloses have few very distinct properties. They are dextrorotatory, have no reducing power, but are very easily hydrolysed to reducing sugars. Some yield furfural and carbon dioxide on heating with hydrochloric acid, but the amounts are variable.

(c) *Estimation.*—Since the properties of the hemicelluloses show such wide variation, it is difficult to devise a satisfactory method of estimation. Methods based on estimation of reducing sugars after hydrolysis with 1% acid are unreliable. The method suggested by Preece (*Biochem. J.* 1931, **25**, 1304) which has been used by the writer, seems the most satisfactory so far introduced. It involves the actual separation and weighing of the hemicelluloses of the tissues, and is admittedly of an empirical nature; it renders possible, however, a more complete analysis of woody tissues than has hitherto been the case. The method is briefly as follows:

25–50 grm. of the material (dried and sieved) are heated in a water-bath for 90 minutes with 300 c.c. of 0.5% ammonium oxalate; the extract is filtered off, and the process repeated. The residue is then heated with 300 c.c. of 50% alcohol, containing 1% of sodium hydroxide, this extraction being subsequently repeated twice. The very dark coloured extract contains the so-called "lignosaccharide." The residue, after filtration, is treated in the cold with 300 c.c. of 4% sodium hydroxide, being allowed to stand for at least 6 hours. The extract is filtered off on a Buchner funnel, through a

circle of stout calico which has been washed thoroughly with 4% sodium hydroxide. The extraction is repeated with further 150 c.c. portions of 4% sodium hydroxide, until the filtrate no longer gives a precipitate on neutralisation with acetic acid, and addition of an equal volume of acetone. The combined extracts contain the "free hemicellulose." The residue left from these extractions is further treated with boiling 4% sodium hydroxide, till again no precipitate results on addition of acetic acid and acetone to the extract; this fraction is termed "combined hemicellulose." (The terms "free" and "combined" are taken only to indicate a difference in the ease of extraction of the substances.) The extracts containing the hemicelluloses are neutralised with acetic acid, and precipitated with excess of acetone; the precipitated hemicellulose is dried with acetone, and finally over phosphorus pentoxide *in vacuo* to a constant weight. Ash and protein are allowed for, and the balance is returned as "hemicellulose." The original paper suggests a scheme for the complete analysis of woody tissues, and should be consulted for further details.

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With Special Consideration of Ante-mortem and Post-mortem Inspection of Food-producing Animals.

By RICHARD EDELMANN, Ph.D., Professor at the Royal Veterinary *High School, Dresden.* Authorised Translation by J. R. MOHLER, A.M., V.M.D., and A. EICHHORN, D.V.S. Fifth Edition. 5 Coloured Plates and 161 Illustrations. 468 pp. 8vo. 28s. *(1925)*

THE COMPARATIVE ANATOMY OF THE DOMESTICATED ANIMALS

By A. CHAUVEAU, M.D., LL.D., and S. ARLOING. Translated and Edited by GEORGE FLEMING. Second Edition. 585 Engravings. 1,084 pp. 8vo. 35s. (1891)

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